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Short Communications

Molecular Characterization and Expression of the Capsid Protein of a Norwalk-like Virus Recovered from a Desert Shield Troop with Gastroenteritis

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Abstract

Norwalk virus (NV) infection was recently found to be associated with gastroenteritis in U.S. military troops stationed in Saudi Arabia during the 1990 Desert Shield Operation. We identified a Norwalk-like virus in the stools of two military personnel with gastroenteritis by ELISA and IEM. By RT-PCR and sequence analysis, the nucleotide sequence of part of the polymerase region of each of these two Desert Shield strains (DSV275 and DSV395) was found to be 73% identical to the corresponding region of NV. In addition, one of the strains (DSV395), which underwent sequence analysis of approximately 2900 consecutive bases, had a genomic organization characteristic of the Caliciviridae. Comparison of the DSV395 amino acid sequence of the capsid region with that of three other viruses in the Norwalk group (Norwalk, Southampton, and Toronto viruses) showed amino acid identity of 47-68%. Consensus sequence analysis of these capsid proteins identified two regions of conserved amino acids that flanked an area of variable amino acids. In addition, the proteins corresponding to the capsid regions of DSV395 and NV were expressed in an in vitro translation system. Immunoprecipitation studies using the expressed capsid proteins and paired DSV395 or NV infection sera indicated the presence of shared antigenic sites between the capsid proteins of DSV395 and NV. However, hyperimmune sera specific for the self-assembled recombinant NV capsid protein did not react with DSV stool antigen in an ELISA, suggesting that there may also be unique antigenic sites not shared between DSV395 and NV.

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Expression and Self-Assembly of Recombinant Capsid Protein from the Antigenically Distinct Hawaii Human Calicivirus

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The Norwalk and Hawaii viruses are antigenically distinct members of the family *Caliciviridae* and are considered to be important etiologic agents of epidemic gastroenteritis, with most studies focusing on the role of Norwalk virus. To further investigate the importance of Hawaii virus, Hawaii virus-like particles (VLPs) were produced by expression of its capsid protein in the baculovirus system and these VLPs were used as the antigen in an enzyme-linked immunosorbent assay that was efficient in the detection of a serologic response to Hawaii virus. The ready availability of Hawaii VLPs should enable larger-scale epidemiological studies to further elucidate the importance of this agent.

Norwalk virus (NV) and NV-like viruses are small, noncultivatable viruses in the family *Caliciviridae* that are major etiologic agents of epidemic gastroenteritis in adults and older children (17). The NV-like human caliciviruses are comprised of at least four distinct serotypes represented by NV, Hawaii virus (HV), Snow Mountain virus (SMV), and Taunton virus, which have been defined by immune electron microscopy (IEM). HV was recovered from a family outbreak of gastroenteritis (29). Cross-challenge studies in adult volunteers, with NV and HV, as well as serologic studies by IEM, have established that these two viruses are antigenically distinct (5, 15, 32). In addition, such volunteer studies have generated important reference reagents for the NV and HV strains that have been used to establish the specificity of new recombinant-protein-based diagnostic assays in the absence of a cell culture system (8, 13, 14, 26, 30). Recent sequence comparisons of the RNA-dependent RNA polymerase (64% amino acid identity) and capsid-encoding (48% amino acid identity) regions of the genomes of NV and HV were consistent with the antigenic differences observed in earlier studies (22). In addition, by sequence analysis of various circulating strains, the human caliciviruses associated with epidemic gastroenteritis have been assigned to either genogroup I (NV related) or genogroup II (SMV or HV related) (reviewed in reference 20). A third genetically distinct group of human caliciviruses represented by the Sapporo and Manchester viruses has also been described (20). However, the full extent of the genetic and antigenic diversity among the human viruses in the family *Caliciviridae* has not been elucidated.

The epidemiology of NV is the most thoroughly investigated of the human caliciviruses (17). Early studies that established the association of NV with epidemic gastroenteritis employed diagnostic assays that relied on viral antigens present in stool specimens obtained from volunteers infected with NV (9, 10). The recent molecular characterization of NV (12) and other human calicivirus (4, 19, 25) genomes has facilitated the development of recombinant DNA-based diagnostic techniques (4, 13, 14, 24). Expression of the capsid protein of NV in the baculovirus system resulted in the production of recombinant NV (rNV) virus-like particles (VLPs) that were similar in efficiency, sensitivity, and specificity to native NV for the detection of serologic responses to NV by both enzyme-linked immunosorbent assay (ELISA) and IEM (8, 13, 26, 30). The availability of an unlimited supply of recombinant antigen for the detection of serological responses to NV has enabled recent large-scale epidemiologic studies that have confirmed the role of NV in gastrointestinal illness in various settings (7, 11, 28). The purpose of our present study was to develop HV-specific diagnostic assays to elucidate the role of HV or HV-related viruses in gastrointestinal disease.

A cDNA copy of ORF2, encoding the capsid protein of HV (HCV/Hawaii/71/U.S.), was generated in a first-round reverse transcription-PCR using a viral RNA template extracted from the stool specimen of an adult volunteer as previously described (22). A second PCR that utilized primers 5′GGATCC GTTTTGTGAATGAAGATG3′ (the 5′ end of ORF2 and a *Bam*HI restriction enzyme site) and 5′GCGGCCGCATTAC TGCACTCTTCT3′ (complementary to the 3′ end of ORF2 with a *Not*I restriction enzyme site) was used to generate an approximately 1,600-bp fragment that was cloned into plasmid pCRII by using the TA system (Invitrogen). The resulting plasmid (designated pCRII-HVORF2) was digested with *Bam*HI and *Not*I, and the ORF2 DNA fragment was purified from an agarose gel and ligated into the compatible sites of the pVL1393 transfer plasmid (Invitrogen) downstream from the baculovirus polyhedrin promoter. Plasmid pVL1393-HVORF2 was cotransfected with linearized baculovirus DNA (BaculoGold; Pharmingen) into Sf9 cells. Recombinant viruses were amplified and screened for the production of VLPs by direct electron microscopy, and selected positive recombinant viruses were plaque purified twice. For production of recombinant HV (rHV) VLPs, recombinant baculoviruses were inoculated onto Sf9 monolayers at a multiplicity of infection of 5 to 10 and particles were purified in 10 to 50% sucrose gradients from the culture fluid of infected cells. VLPs obtained by expression of the HV capsid protein in the baculovirus system were morphologically similar to previously described NV VLPs (13) (Fig. 1). By negative-stain electron microscopy, numerous approximately 27- to 35-nm particles were observed, some of which appeared empty and others of which appeared full. Subviral-size (approximately 20-nm) particles (not shown) were also

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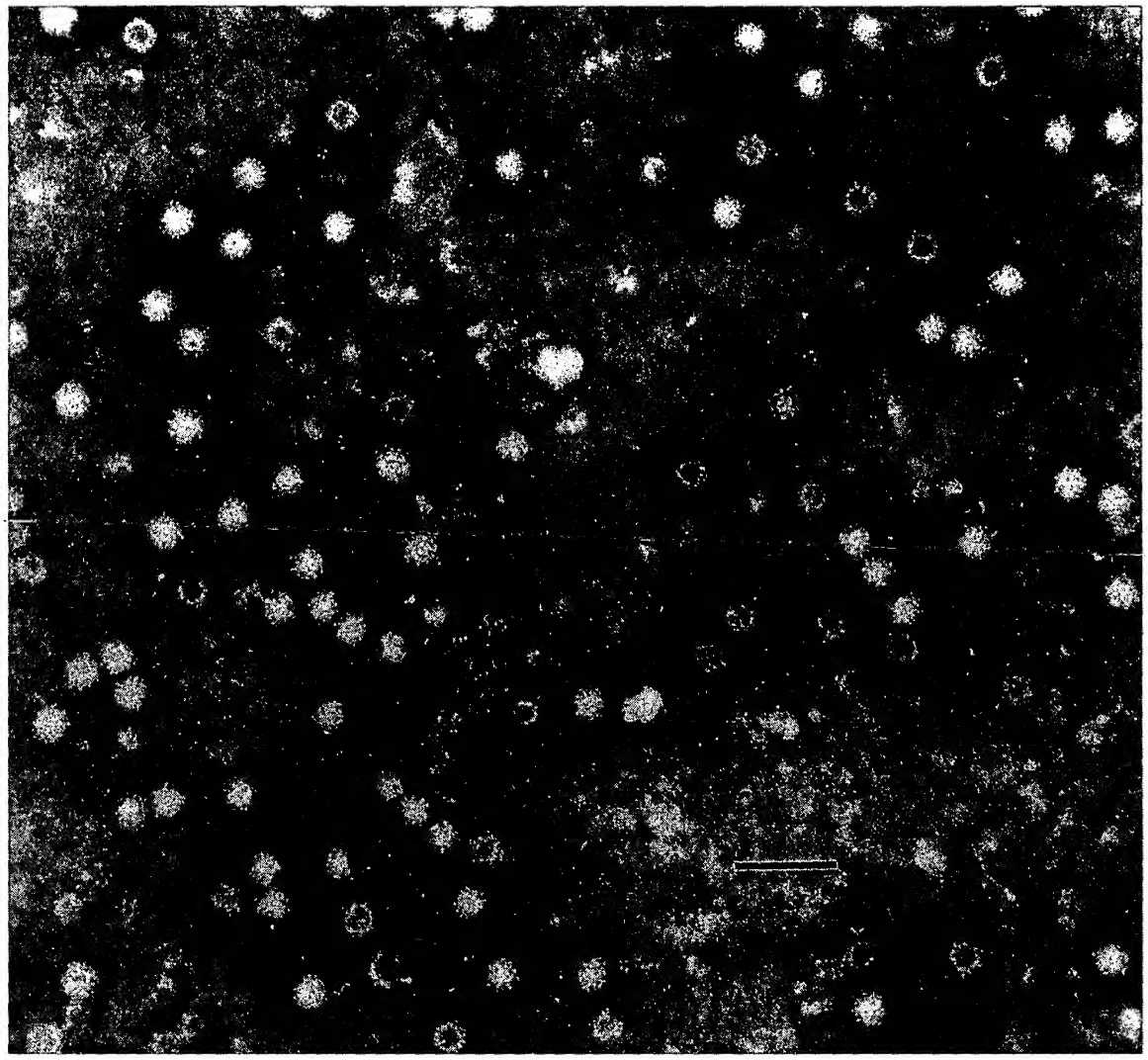

FIG. 1. Purified HV-like particles expressed in the baculovirus system and visualized by negative staining with 3% phosphotungstic acid, pH 7.2. Bar, 100 nm.

observed variably. Hyperimmune sera specific for the rHV VLPs were generated by immunization of guinea pigs with purified VLPs by using standard protocols, and these sera reacted with the rHV protein by ELISA and Western blotting (data not shown).

The rHV VLPs were used as an antigen in the development of an ELISA that was performed in a format similar to that previously described by Jiang et al. for rNV (13). Briefly, purified rHV VLPs were diluted in phosphate-buffered saline and adsorbed to the wells (1 μg/well) of a polyvinyl microtiter plate by incubation overnight at 4°C. Following blocking of nonspecific binding sites on the plate with 5% BLOTTO (nonfat dry milk in phosphate-buffered saline), serial twofold dilutions of each serum sample were made at a starting dilution of 1:50. The binding of antibodies to the rHV antigen was detected with peroxidase-conjugated goat anti-human antibody (Cappel) and 2,2′-azinobis(3-ethylbenzthiazolinesulfonic acid) (ABTS) substrate. Titers were reported as the reciprocal of the highest dilution that met the definition for a positive reaction with the rHV antigen (an optical density at 405 nm of ≥0.200). A significant serologic response was defined as a fourfold or greater increase in the titer of the antibody to the recombinant antigen between preinfection or acute-phase and postinfection sera. Paired sera from an individual were tested simultaneously in duplicate wells. A rNV ELISA (13) was used to detect antibodies to NV by using reagents kindly provided by Mary Estes, Baylor College of Medicine, Houston, Tex.

Paired preinfection or acute-phase and postinfection sera from selected HV, NV, or NV-related virus studies were evaluated for antibodies to rHV to establish the specificity of the rHV ELISA as follows: (i) five adult volunteers who had developed gastrointestinal illness following oral administration of

TABLE 1. Serologic responses of adult volunteers orally challenged with HV to rHV or rNV antigen as measured by ELISA[a]

Individual	Serum sample	Reciprocal titer of antibody to:	
		rHV	rNV[a]
1	Prechallenge	800	3,200
	Postchallenge	**51,200**[b]	6,400
2	Prechallenge	800	800
	Postchallenge	**3,200**	800
3	Prechallenge	800	3,200
	Postchallenge	**25,600**	3,200
4	Prechallenge	<400	1,600
	Postchallenge	**1,600**	800
5	Prechallenge	50	400
	Postchallenge	**25,600**	400

[a] rNV data are from Green et al. (8). Volunteers challenged with HV stool filtrate or HV passaged in volunteers also failed to develop a serologic response to native NV by IEM (5, 15).

[b] Values in boldface indicate serologic response as defined in the text.

HV (32) (Table 1); (ii) three chimpanzees that were challenged with NV by the alimentary route and developed antibody responses to native NV by IEM and to rNV as previously reported (8, 33), (no. 1 to 3 in Table 2); (iii) two adult volunteers challenged orally with the Montgomery County agent, a virus antigenically related to NV (15, 29) (no. 4 and 5 in Table 2); and (iv) a member of the U.S. military infected under natural conditions with the Desert Shield strain (HCV-DSV395/90/Saudi Arabia), a virus belonging to NV-related genogroup I (21) (no. 6 in Table 2). Thus, the rHV ELISA detected evidence of infection with native HV efficiently but did not detect evidence of infection with NV or NV-related virus. These findings were consistent with previous reports that the rNV ELISA did not efficiently detect serologic evidence of infection in volunteers challenged with HV (8, 30).

The specificity of the rHV ELISA was also evaluated by using paired sera from adult volunteers who underwent oral challenge with SMV strain HCV/SMV/76/U.S., a reference strain that has been identified as antigenically distinct from NV and HV by IEM and belonging to genogroup II (as does HV) by sequence analysis of the polymerase region (6, 31). The polymerase region of the SMV genome shares 59 and 92% amino acid identity with NV and HV, respectively (22, 31). Four of five adult volunteers challenged with SMV and previously shown to develop a serologic response to this virus in a blocking antibody immunoassay (BAI) (26) failed to develop a serologic response to either rHV or rNV by ELISA (no. 7 to 10 in Table 2). The characteristic inefficiency of the rHV ELISA in detecting significant antibody responses to SMV was consistent with the identification of HV and SMV as distinct serotypes by IEM and suggests that a strong genetic relatedness in the polymerase region does not necessarily reflect antigenic relatedness in the capsid protein. However, one individual (no. 11 in Table 2) infected with SMV developed a serologic response to both rHV and rNV. In addition, a pediatric patient in a Canadian hospital undergoing natural infection with the Toronto 19 strain (HCV/TV19/90/CAN), belonging to genogroup II (23), showed a serologic response to both rNV and rHV (no. 12 in Table 2). The cross-reactive responses to both NV and HV observed in individuals 11 and 12 in Table 2 are of interest because each of them underwent infection with viruses that have been genetically characterized as belonging to genogroup II. It is not known whether these cross-reactive responses reflect evidence of prior calicivirus infection in these individuals or the current infection. These occasional broad antibody responses indicate that the analysis of paired sera from individuals undergoing natural infection with circulating viruses must be interpreted with caution in regard to the antigenic specificity of the infecting strain.

The relationship of the rHV was examined with paired sera derived from three other well-characterized gastroenteritis outbreaks that were not associated with NV in previous studies. Four adult volunteers who were orally administered the Wollan or W agent (16), which was associated with a gastroenteritis outbreak in an English boarding school (3), and who developed illness following the challenge were examined for serologic responses to the rHV. Two of the four developed a serologic response to rHV by ELISA, whereas none of them developed a response to rNV or to NV in each of the three tested by IEM (no. 13 to 16 in Table 2) (16). Paired sera from four individuals involved in the Henryton II and Morgantown, W.Va., outbreaks (1, 2) were examined (no. 17 to 20 in Table 2). An agent(s) associated with the Morgantown, W.Va., outbreak could not be identified in this serologic analysis. However, it is possible that the Wollan and Henryton II outbreaks were caused by a human calicivirus. Further characterization of these viruses is needed to address this possibility.

Serologic responses to rHV were examined in 223 individuals involved in 23 outbreaks of nonbacterial gastroenteritis that were not associated with NV in a previous study (27). Twenty-three (10.3%) of the individuals examined showed a fourfold or greater increase in antibody to HV (Table 3), and as previously reported (27), only 3 (1.4%) of 207 developed a serologic response to NV in the NV BAI ELISA. Two of the outbreaks met the definition for an HV outbreak established previously, i.e., 50% or more of the affected individuals showed a serologic response to the assayed agent (9, 18). Furthermore, 14 (61%) of these 23 outbreaks included one or more individuals who developed a serologic response to rHV, suggesting that a human calicivirus distinct from both NV and HV may have been the primary cause of illness in these outbreaks. As assays become available for other antigenically distinct human caliciviruses, the identification of caliciviruses as etiologic agents of epidemic gastroenteritis will undoubtedly increase.

NV has been established as a cause of nonbacterial epidemic gastroenteritis, but few studies have examined the role of HV. Expression of ORF2 of HV in the baculovirus system resulted in the production of VLPs that were antigenically indistinguishable from native HV, and these VLPs were used in the development of an ELISA for the detection of HV-specific antibodies. Moreover, a dissociation of serologic responses to NV and HV was observed when paired sera from challenge studies were analyzed, consistent with the major antigenic differences between these two viruses that had been established in cross-challenge studies and by IEM. The extent of antigenic variation among circulating human calicivirus strains associated with epidemic gastroenteritis is an important characteristic of these viruses that is relevant for an understanding of their epidemiology and natural history. Several classification systems for human calicivirus serotypes have been proposed, but the limited quantities of reference stool specimens containing the native virus have impeded the development of standardized reagents. The availability of recombinant VLPs for major reference strains such as NV and HV, the first two distinct serotypes described, should facilitate the development of such reagents.

TABLE 2. Comparison of serologic responses to rHV and rNV in adults or chimpanzees infected with various agents of gastroenteritis

Human or chimpanzee	Source of serum	Serum sample	Reciprocal titer of antibody to:	
			rHV	rNV[a]
1	NV challenge (chimpanzee 14G)	Prechallenge	400	1,600
		Postchallenge	200	**≥102,400**[b]
2	NV challenge (chimpanzee 15G)	Prechallenge	400	400
		Postchallenge	200	**409,600**
3	NV challenge (chimpanzee 16G)	Prechallenge	<50	<50
		Postchallenge	<50	**6,400**
4	Montgomery County agent challenge	Prechallenge	100	400
		Postchallenge	100	**3,200**
5	Montgomery County agent challenge	Prechallenge	200	1,600
		Postchallenge	200	800
6	Desert Shield virus (DSV395), U.S. military	Preinfection	3,200	400
		Postinfection	1,600	**6,400**
7	SMV challenge (86-23)	Prechallenge	6,400	400
		Postchallenge	12,800	400
8	SMV challenge (86-24)	Prechallenge	1,600	1,600
		Postchallenge	3,200	1,600
9	SMV challenge (86-26)	Prechallenge	400	400
		Postchallenge	400	200
10	SMV challenge (86-27)	Prechallenge	800	800
		Postchallenge	1,600	1,600
11	SMV challenge (86-25)	Prechallenge	400	≤50
		Postchallenge	**12,800**	**200**
12	Toronto virus (TV19) nosocomial outbreak	Acute phase	<50	<50
		Postinfection	**800**	**200**
13	Wollan agent challenge	Prechallenge	6,400	400
		Postchallenge	3,200	200
14	Wollan agent challenge	Prechallenge	1,600	1,600
		Postchallenge	**6,400**	1,600
15	Wollan agent challenge	Prechallenge	400	200
		Postchallenge	800	200
16	Wollan agent challenge	Prechallenge	400	50
		Postchallenge	**1,600**	100
17	Henryton II outbreak	Acute phase	3,200	3,200
		Postinfection	3,200	3,200
18	Henryton II outbreak	Acute phase	3,200	3,200
		Postinfection	**12,800**	3,200
19	Morgantown, W. Va., outbreak	Acute phase	400	800
		Postinfection	400	800
20	Morgantown, W. Va., outbreak	Acute phase	400	3,200
		Postinfection	400	1,600

[a] Data for rNV titers are from reference 8 (chimpanzees 1 to 3 and humans 4, 5, 13 to 15, and 17 to 20) or references 21 and 23 (humans 6 and 12, respectively).
[b] Data in boldface indicate a serologic response as defined in the text.

TABLE 3. Summary of serologic responses to rHV in individuals involved in 23 outbreaks of gastroenteritis not associated with NV

Location of outbreak	Setting	Date of outbreak (mo/yr)	No. of individuals with ≥4-fold response to indicated virus (assay)/no. (%) tested	
			HV (rHV ELISA)	NV (BAI)[a]
At sea	Cruise ship	9/76	0/4	0/4
New York	Hospital	11/76	5/18 (28)	0/19
North Carolina	College	3/77	1/7 (14)	0/8
Wyoming	Recreational area	7/77	0/7	1/7 (14)
Vermont	Nursing home	1/78	**3/6 (50)**[b]	1/3 (33)
Florida	Nursing home	2/78	1/25 (4)	0/25
Missouri	Community	5/78	1/6 (17)	0/6
At sea	Cruise ship	1/79	1/8 (13)	0/10
At sea	Cruise ship	2/79	2/10 (20)	0/12
Florida	Hospital	5/79	0/15	0/5
Alaska	Family	6/79	1/13 (8)	0/13
Virginia	Swimming pool	7/79	1/11 (9)	0/7
Hawaii	Restaurant	1/80	1/14 (7)	0/9
Florida	Nursing home	3/80	1/6 (17)	0/4
California	Restaurant	5/80	0/9	0/8
Illinois	College	5/80	**3/5 (60)**	1/7 (14)
Illinois	Restaurant	5/80	1/12 (8)	0/12
Washington	Hospital	5/80	0/7	0/7
Colorado	Nursing home	6/80	0/1	0/6
New York	Camp	7/80	1/14 (7)	0/4
Florida	Hospital	7/80	0/9	0/10
New York	Restaurant	4/82	0/7	0/7
Wisconsin	Hospital	6/82	0/9	0/14
Total			23/223 (10.3)	3/207 (1.4)

[a] Data are from Midthun et al. (27), who used the NV BAI, an assay that employed NV antigen in stool and is similar in efficiency and specificity to the rNV ELISA (8).

[b] Data in boldface indicate an outbreak in which ≥50% of the individuals developed a serologic response to the assayed virus.

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Biochemical Characterization of a Smaller Form of Recombinant Norwalk Virus Capsids Assembled in Insect Cells

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The expression of the single capsid protein of Norwalk virus (NV) in *Spodoptera frugiperda* (Sf9) insect cells infected with recombinant baculovirus results in the assembly of virus-like particles (VLPs) of two sizes, the predominant 38-nm, or virion-size VLPs, and smaller, 23-nm VLPs. Here we describe the purification and biochemical characterization of the 23-nm VLPs. The 23-nm VLPs were purified to 95% homogeneity from the medium of Sf9 cultures by isopycnic CsCl gradient centrifugation followed by rate-zonal centrifugation in sucrose gradients. The compositions of the purified 23- and 38-nm VLPs were compared by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and protein immunoblots. VLPs of both sizes showed a doublet at 58 kDa, the size of the full-length capsid protein. Upon alkaline treatment, the 23-nm VLPs underwent dissociation into soluble intermediates that were able to reassemble into 23- and 38-nm VLPs upon dialysis, suggesting that the assembly of both types of structures has a common pathway. Antigenic and biochemical properties of the 38- and 23-nm VLPs were examined and found to be conserved. Immunoprecipitation assays using polyclonal and monoclonal antibodies indicated that immunodominant epitopes on the capsid protein as well as conformational epitopes are conserved in the two types of particles. The trypsin cleavage site at residue 227 was protected in the assembled particles of both sizes but exposed after alkaline dissociation. These results, and the conservation of the binding activity of both forms of recombinant NV VLPs to cultured cells (L. J. White, J. M. Ball, M. E. Hardy, T. N. Tanaka, N. Kitamoto, and M. K. Estes, J. Virol. 70:6589–6597, 1996), suggest that the tertiary folding of the capsid protein responsible for these properties is conserved in the two structures. We hypothesize that the 23-nm VLPs are formed when 60 units of the NV capsid protein assembles into a structure with T=1 symmetry.

Norwalk virus (NV) is a prototype strain of human caliciviruses, a group of pathogens responsible for outbreaks of gastroenteritis in humans (8). Norwalk virus is a small icosahedral virus composed of a single structural protein that contains a single-stranded plus-sense RNA genome of about 7.7 kb (20). This single polypeptide must encode all the functional entities required for NV structural integrity, infectivity, and immunogenicity. Study of this group of viruses has been hampered by the low amounts of virus in stool samples from infected individuals, along with the absence of a cell culture system and an animal model to propagate the virus (8).

The capsid proteins from several strains of human caliciviruses including NV (18), Mexico virus (17), Lordsdale virus (5), Toronto virus (21), Snow Mountain agent (11), and human calicivirus Sapporo (22) have been expressed in insect cells infected with recombinant baculoviruses. The capsid proteins from these viruses spontaneously assemble into empty virus-like particles (VLPs) that are released into the medium of the insect cell cultures from which they can be purified usually in high yields. In preparations of recombinant NV (rNV) VLPs (28) and recombinant Mexico virus VLPs (17) expressed in insect cell cultures, two sizes of particles have been found; the major fraction has a diameter of approximately 38 nm as determined by electron cryomicroscopy (24), while a small proportion of particles has a smaller diameter reported to be approximately 19 nm (28), 15 to 20 nm (17), or 23 nm (this study; see below). Particles of two sizes (35 to 40 and 15 to 20 nm) were also observed by immunoelectron microscopy in stool samples from patients infected with Otofuke agent (a human calicivirus present in stools during an outbreak of diarrhea in Japan) (27). The 38-nm rNV VLPs have been well characterized antigenically and morphologically (18), and the three-dimensional structure has been determined to a resolution of 2.2 nm by electron cryomicroscopy and computer image processing techniques (24). These studies revealed that the 38-nm rNV VLPs fold into T=3 icosahedral structures formed by 180 copies of the capsid protein. There are 90 arch-like capsomers at the local and strict twofold axes.

The self-assembly of viral capsids composed of a single structural protein into more than one structure has been described for icosahedral T=3 RNA plant viruses. The capsid proteins from southern bean mosaic virus (SBMV) (16, 25), turnip crinkle virus (26), alfalfa mosaic virus (9), and brome mosaic virus (4) are able to self-assemble in vitro into T=3 or smaller T=1 structures depending on factors such as trypsin digestion, the presence and size of the RNA genome, the presence of divalent cations, the ionic strength, and the pH.

The involvement of only one structural protein in the formation of the NV capsids, along with the availability of high-resolution structural data (24) and the ability of the capsid protein to dissociate and reassemble in vitro (14; also, this study), makes NV a good tool for morphogenesis studies of small icosahedral animal viruses. The biochemical and structural characterization of different forms of empty rNV VLPs may be helpful for evaluating the protein-protein interactions that govern assembly independently from the influence of protein-RNA interactions.

This paper describes the purification and biochemical characterization of the smaller 23-nm rNV VLPs assembled in vivo in insect cell cultures. Specifically, we analyzed the protein

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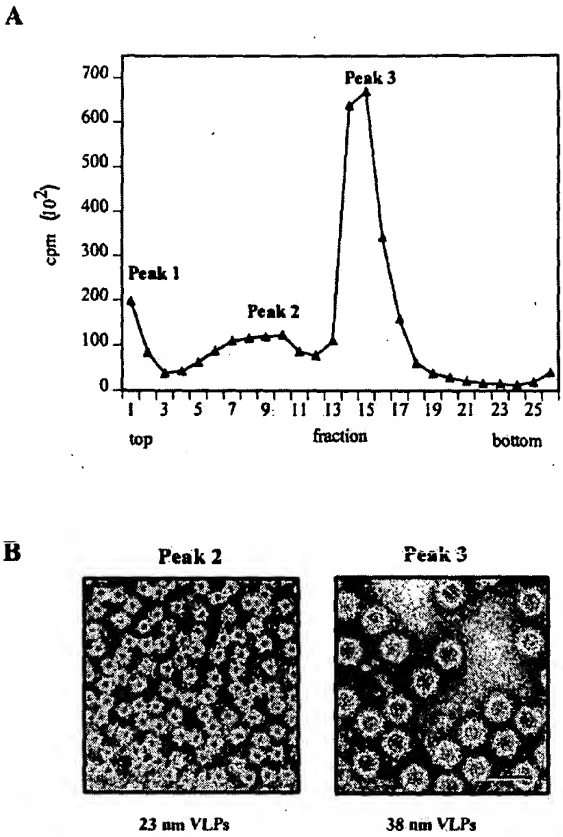

FIG. 1. Sedimentation of rNV VLPs on sucrose gradients. Preparations of VLPs separate into soluble capsid protein (peak 1), 23-nm VLPs (peak 2), and 38-nm VLPs (peak 3). (A) ^{35}S-labeled rNV VLPs were pelleted for 2 h at 26,000 rpm in a Beckman SW28 rotor at 4°C from the medium of infected Sf9 cell cultures 7 days after infection and suspended in 0.01 M PBS, pH 7.2. The VLPs were then centrifuged in a CsCl isopycnic gradient and banded at a density of 1.31 g/cm^3. The VLPs collected in the band were pelleted for 2 h at 26,000 rpm in a Beckman SW28 rotor at 4°C and then sedimented in a five-step discontinuous 5 to 25% (wt/vol) sucrose gradient for 3.5 h at 35,000 rpm in a Beckman SW41 rotor at 4°C. Fractions were collected from the top, and radioactivity from each fraction was measured by liquid scintillation spectrometry. (B) Electron micrographs of purified particles in peaks 2 and 3. Fractions forming peaks 2 and 3 were pooled separately and concentrated by pelleting for 2 h at 26,000 rpm in a Beckman SW28 rotor at 4°C. Pelleted rNV VLPs were suspended in 0.01 M PBS, pH 7.2, and analyzed by negative-stain electron microscopy. Carbon-coated collodion 400-mesh grids were layered on top of 10 μl of the sample on Parafilm and incubated for 10 min at room temperature; excess fluid was removed with the edge of a filter paper disk, and the grids were washed with 10 μl of milli-Q water and stained for 30 s with 1% ammonium molybdate, pH 5.5. Bar, 50 nm.

composition, susceptibility to protease treatment, surface and antigenic properties, and the products of in vitro dissociation-reassembly of the 23-nm VLPs compared to the 38-nm VLPs.

Purification of 23- and 38-nm VLPs. Metabolically radiolabeled (Trans ^{35}S-label; ICN, Irvine Calif.) 23- and 38-nm VLPs were purified separately from the media of Sf9 cell cultures infected with recombinant baculovirus (Bac-rNV C8) (18). Sf9 cells were infected and the culture medium was harvested as described earlier (28). The VLPs were concentrated from the media by ultracentrifugation followed by isopycnic CsCl gradient centrifugation (1.362 g/cm^3), where particles of both sizes banded at the same density of 1.31 g/ml. This mixed population of rNV VLPs contained predominantly 38-nm particles. The proportion of smaller-size VLPs varied from one preparation to another, being absent or undetectable in some preparations and representing up to 30% of the particles in others. To separate the two sizes of particles, the VLPs in the CsCl band were concentrated by ultracentrifugation and separated by rate-zonal centrifugation on a discontinuous 5 to 25% (wt/vol) sucrose gradient. The gradients were fractionated, and aliquots of each fraction were analyzed by scintillation counting. Three peaks of radioactivity were detected (Fig. 1A). The fractions forming peaks 2 and 3 were pooled separately, and the VLPs were concentrated by ultracentrifugation and suspended in 0.01 M PBS, pH 7.2. Aliquots from each of the three peaks were prepared for electron microscopic analysis by negative staining with 1% ammonium molybdate, pH 5.5. The diameters of the rNV VLPs were measured in electron microscopic negatives at a magnification of ×39,000. The material in peak 1, located at the top of the gradient, did not contain any particles detectable by electron microscopy, in agreement with peak 1 being soluble protein. Peak 2 contained VLPs with a diameter of 23.3 ± 0.73 nm, and peak 3 contained VLPs with a diameter of 34 ± 1.97 nm (Fig. 1B). By electron cryomicroscopy, the larger VLPs measured 38 nm (24). Electron microscopy analysis by negative-stain procedures gives particle sizes smaller than those observed by high-resolution structural analysis (23). Based on this, we predict that the actual size of the small particles measured by electron cryomicroscopy will be 26 to 27 nm. The sizes reported earlier for the smaller rNV VLPs by negative-stain techniques were 19 nm (28) and 15 to 20 nm (17), and the smaller "empty" particles in stool samples from patients infected with Otofuke agent were 15 to 20 nm (27). The difference between these sizes and our current measurement of 23 nm may be related to differences in the stain used, its concentration, and pH. The overall morphology of the 23-nm VLPs was similar to that of the 38-nm VLPs. The particles appeared empty, with protrusions similar to those in the images of 38-nm VLPs.

Both 23- and 38-nm VLPs are composed of the 58K capsid protein. To determine whether the smaller capsids were

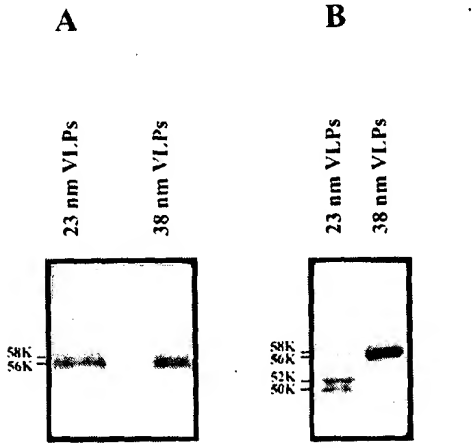

FIG. 2. VLPs with a diameter of 38 or 23 nm are composed predominantly of the 58K capsid protein. (A) The protein composition of VLPs purified as indicated in the legend to Fig. 1 was determined by SDS–10% PAGE. Protease inhibitors (aprotinin and leupeptin at 0.5 μg/ml each) were used throughout the purification procedure. Samples were boiled for 2 min, and equal amounts of radioactivity (counts per minute) were loaded onto the gel. Bands were visualized by autoradiography. (B) Protein analysis by SDS–12% PAGE of 23- and 38-nm VLPs purified and stored in the absence of protease inhibitors.

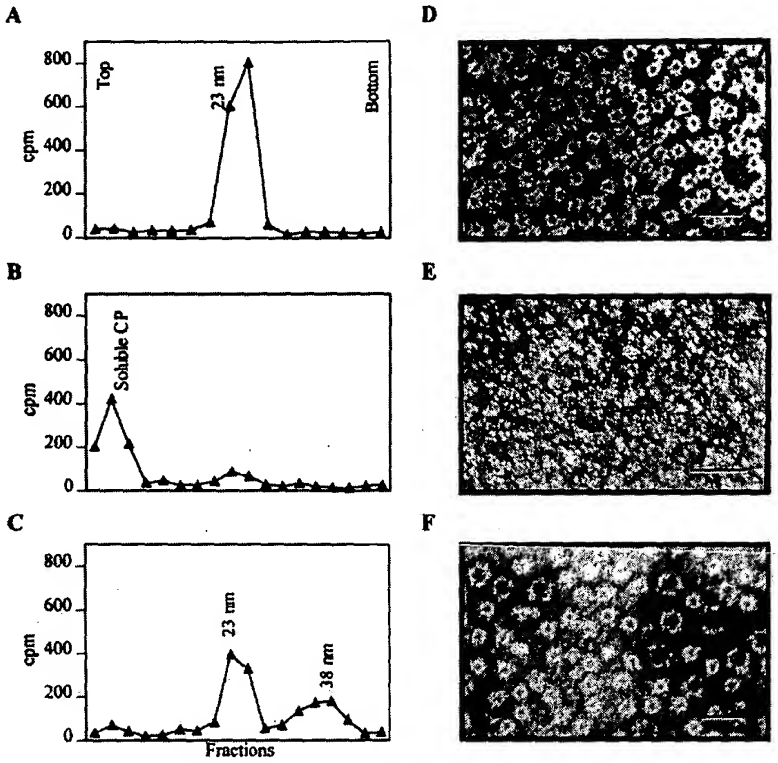

FIG. 3. In vitro disassembly-reassembly of 23-nm VLPs makes 23- and 38-nm VLPs. (A to C) Rate-zonal centrifugation on a discontinuous 5 to 25% (wt/vol) sucrose gradient made in 0.1 M phosphate buffer, pH 6.0 (A and C) or in 50 mM Tris-HCl, pH 8.9 (B) was used to characterize the products of the disassembly and reassembly of the 23-nm VLPs. The gradients were centrifuged for 1.5 h in a Beckman TLS55 rotor at 35,000 rpm at 4°C. Fractions were collected by bottom puncture, and the radioactivity was quantitated by scintillation counting. (A) Twenty-three-nanometer VLPs purified as indicated in the legend to Fig. 1; (B) purified 23-nm VLPs after treatment with 50 mM Tris, pH 8.9, at 4°C overnight; (C) alkaline-treated 23-nm VLPs after dialysis against 0.1 M phosphate buffer, pH 6.0, at 4°C; (D to F) electron micrographs of purified 23-nm VLPs corresponding to the ones characterized in panels A to C, respectively. Negative staining was performed as indicated in the legend to Fig. 1. Bars, 50 nm. CP, capsid protein.

formed by the intact NV capsid protein or by a cleaved or degraded fragment of this protein, 23- and 38-nm VLPs that were purified and stored in the presence of the protease inhibitors aprotinin (Sigma, St. Louis, Mo.) (0.5 μg/ml) and leupeptin (Sigma) (0.5 μg/ml) were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) (Fig. 2A). VLPs of both sizes showed the same protein composition pattern, represented by a doublet at ~58 kDa. The smaller protein in the doublet (~56 kDa) is often seen in purified preparations of 38-nm VLPs, even when protease inhibitors are present during purification, and is likely an early cleavage product of the capsid protein within Sf9 cells. In overexposed gels, the 23-nm VLPs showed two smaller bands with approximate molecular weights of 52,000 and 50,000 (data not shown). The two smaller bands were not present in preparations of 38-nm VLPs purified simultaneously. Upon storage in the absence of protease inhibitors, the capsid protein was less stable in the 23-nm VLPs than in the 38-nm VLPs, as shown by a more rapid degradation of the 58-kDa (58K) protein into the 56K, 52K, and 50K cleavage products (Fig. 2B); these cleavage products, specifically the 52K and 50K products, appeared more slowly in the 38-nm VLPs. The higher instability of the 58K capsid protein in the 23-nm VLPs does not correlate with a higher instability of the particles, which remain intact in phosphate-buffered saline (PBS), pH 7.2, at 4°C for several months. The smaller proteins were related to the NV capsid protein based on reactivity with rabbit anti-rNV sera in Western blot (data not shown). The cleaved fragments reacted with an NV peptide antiserum directed against the C-terminal 15 residues of the NV capsid protein, suggesting that these fragments are cleaved in their N terminus. These observations suggest that the 58K capsid protein in the 23-nm VLPs is more susceptible to degradation of the N-terminal residues than the 58K capsid protein in the 38-nm VLPs.

In vitro disassembly-reassembly of 23-nm VLPs results in 23- and 38-nm VLPs and occurs through common soluble intermediates. To begin to understand the morphogenesis of the 23-nm VLPs, experiments on in vitro dissociation and reassembly of the 23-nm VLPs were performed. Purified 23-nm VLPs (0.3 to 1 mg/ml) were dissociated into soluble intermediates by alkaline treatment (50 mM Tris HCl, pH 8.9; overnight at 4°C) as described previously (13). Complete dissociation of the particles into soluble protein was shown by a shift of the radiolabeled peak of rNV VLPs from the center to the top of a 5 to 25% (wt/vol) sucrose gradient (Fig. 3A and B) and by the disappearance of particles examined by negative-stain electron microscopy (Fig. 3D and E). The soluble capsid protein was then dialyzed against 0.1 M phosphate buffer, pH 6.0, which allows the reassembly of the soluble protein into particles in vitro. Analysis of the dialyzed protein on a 5 to 25% sucrose gradient prepared in 0.1 M phosphate buffer, pH 6, revealed two peaks of radioactivity, corresponding to 23- and

TABLE 1. Biochemical properties of 23- and 38-nm VLPs

Biochemical property	Results with VLPs	
	23 nm	38 nm
Immunoprecipitation by polyclonal RαrNV[a]	Yes	Yes
Immunoprecipitation by MAbs NV8812, NV7411 and NV8301[b]	Yes	Yes
Binding to Caco-2 cells in culture[c]	Yes; competition with 38-nm VLPs	Yes
Trypsin treatment of VLPs	Resistant	Resistant
Trypsin treatment of alkali-dissociated VLPs[d]	32K cleavage product	32K cleavage product

[a] RαrNV, hyperimmune rabbit anti-rNV serum (18).
[b] MAbs NV8812, NV7411, and NV8301 recognize conformational epitopes at the C-terminal 302 residues (12).
[c] Data reported previously (28).
[d] Trypsin cleaves the NV capsid protein at residue 227, yielding a major product of 32K corresponding to the C-terminal residues 228 to 530 (13).

38-nm VLPs (Fig. 3C), as confirmed by electron microscopic analysis of an aliquot of the dialyzed material before sucrose gradient centrifugation (Fig. 3F). SDS-PAGE of the in vitro-reassembled 23- and 38-nm VLPs indicated that the 58K protein and the smaller fragments of the capsid protein were incorporated into the two types of VLPs in the same proportion (data not shown). Since the precise nature of the soluble intermediates in the alkaline dissociation of the 23-nm VLPs is unknown, we do not know whether these cleaved fragments were incorporated as monomers into the growing capsids or as part of larger intermediates that were incompletely disassembled. We currently are investigating the composition of the disassembly intermediates to address this question. However, the reassembly of particles of both sizes suggest that the alkaline treatment of the 23-nm VLPs generates soluble intermediates that are building blocks in the assembly of both 23- and 38-nm VLPs. We hypothesize that these soluble intermediates are oligomers that tolerate N-terminal cleavage of their component capsid proteins probably because those cleavages do not affect dimerization and the protein-protein interactions required for assembly of both types of VLPs. The results also indicate that the conditions used in our reassembly assay were favorable for the formation of both structures. It has been observed that when the 38-nm VLPs are dissociated under the same conditions (data not shown), more than 98% of the reassembled VLPs are 38 nm in diameter, suggesting that the type of VLP that is dissociated affects the ratio of reassembled VLPs. Since the nature and composition of the soluble intermediates may also determine the ratio of reassembled particles, further characterization of the intermediate products of the alkaline dissociation will facilitate understanding of how alternative pathways are selected.

Antigenic and biochemical properties of the 23- and 38-nm VLPs are similar. Having demonstrated that the 58K full-length capsid protein is the major component of both 23- and 38-nm VLPs and that the building blocks in the assembly of both structures are common, we proceeded to examine whether the capsid proteins in the two structures have the same properties regarding antigenic domains and susceptibility to trypsin cleavage. These data should provide some insight into the degree of conservation of the tertiary and quaternary folding of the capsid protein in each structure.

The antigenic properties of the 23-nm VLPs were compared to those of the 38-nm VLPs by analyzing the reactivities of both types of particles with polyclonal and monoclonal (MAb) antibodies to rNV VLPs by immunoprecipitation. The results are summarized in Table 1. Radiolabeled 23- or 38-nm VLPs were immunoprecipitated under nondenaturing conditions with rabbit polyclonal hyperimmune antisera to rNV VLPs that behave as type-specific sera based on the lack of reactivity with NV-related viruses in enzyme-linked immunosorbent assay (19) and with each of the following rNV MAbs: NV8812, NV7411, and NV8301, diluted 1:50 and 1:100 as described elsewhere (28). These MAbs have been shown to recognize discontinuous epitopes on the C-terminal 302 residues of the NV capsid protein (12). Polyclonal antibodies and MAbs were able to immunoprecipitate the 58K capsid protein in both the 38- and the 23-nm particles as well as the cleaved fragments in the 23-nm VLP preparations (data not shown). These results indicate that the immunodominant epitopes on the capsid protein as well as the conformational epitopes recognized by these MAbs are conserved in the two types of particles.

We also tested the susceptibility of the capsid protein in particulate form and in soluble form to trypsin treatment. The NV capsid protein in the 38-nm VLPs is resistant to trypsin cleavage, but if the VLPs are pretreated with alkali to dissociate them into soluble protein, the trypsin cleavage site at residue 227 becomes exposed, and trypsin cleavage at residue 227 results in a 32K fragment of residues 228 to 530 (13). ^{35}S-radiolabeled 38- and 23-nm VLPs (0.2 μg/μl) were treated for 5 min with 10 mM Tris, pH 8.9, at room temperature to partially dissociate the VLPs. The particles were then subjected to sucrose gradient centrifugation to separate the soluble capsid protein from the protein assembled in VLPs. Two peaks of radioactivity corresponding to dissociated (banding at the top of the gradient) and particulate (banding at the middle of the gradient) NV capsid protein were detected in gradients of both 23- and 38-nm VLPs. Aliquots from each peak were treated with 10 μg of *N*-tosyl phenylalanine chloromethyl ketone (Worthington Biochemical, Freehold, N.J.) for 30 min at 37°C and then analyzed by SDS-PAGE. The capsid protein in the 23-nm VLPs showed the same behavior upon trypsin treatment as did the 38-nm VLPs (Table 1). The capsid protein was resistant to cleavage when in a particle, while the dissociated protein was susceptible to digestion, yielding a 32K cleavage product. These results indicate that the trypsin cleavage site at position 227 is protected in both structures, becoming exposed only after alkaline dissociation of VLPs.

We have demonstrated previously that the domain on the capsid protein responsible for specific binding to cells in culture (residues 300 to 384) is conserved in the two structures by showing that purified 23-nm VLPs were able to compete with the 38-nm VLPs for specific binding sites on the surface of differentiated human intestinal cells (Caco-2) grown in cultures (28). The conservation of conformational antigenic domains, trypsin cleavage susceptibility, and cell binding activity suggests that the capsid protein in the small particles may fold into a tertiary structure similar to the 38-nm VLPs. Atomic-resolution structural data will confirm this hypothesis.

The data presented in this paper indicate that the capsid

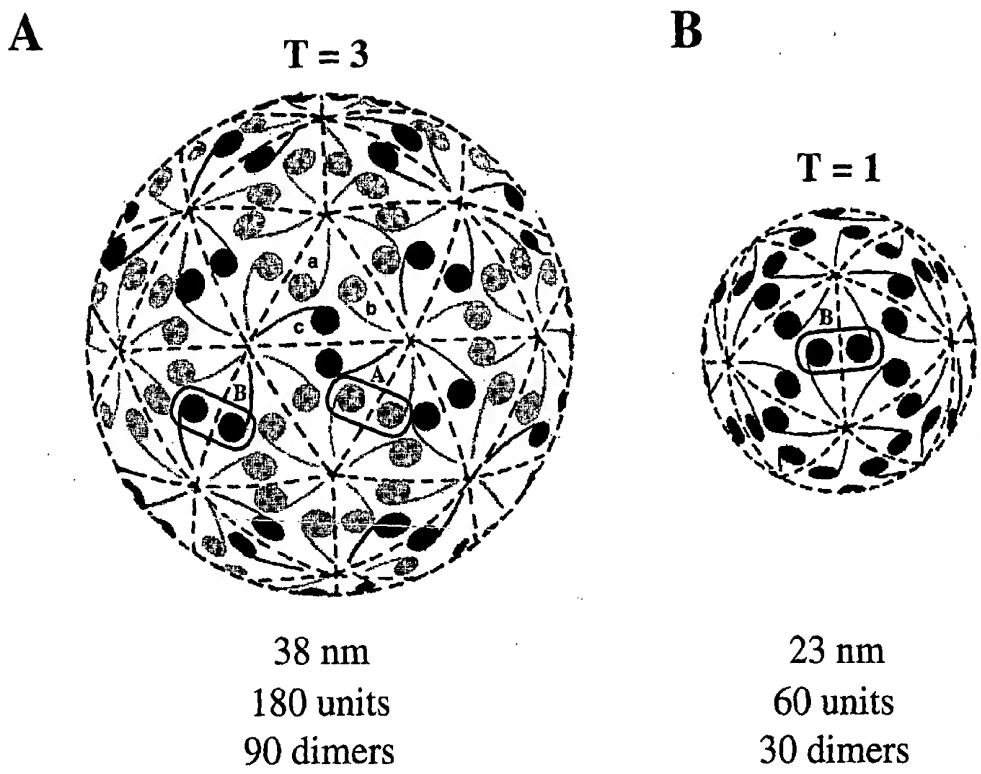

FIG. 4. Schematic representation of virus capsids with T=3 and T=1 icosahedral symmetries. (A) The T=3 capsid structure of the 38-nm rNV VLPs and other single-stranded RNA small spherical viruses (16) is formed by 180 molecules of the capsid protein; each protein molecule is represented by a comma-like symbol. The subunits are chemically identical, but intersubunit contacts differ depending on subunit location in the shell (a, b, or c). The 180 subunits are arranged as 90 dimers of two types: 60 A dimers (quasiequivalent; shaded commas) surround the icosahedral fivefold axis, and 30 B dimers (equivalent; solid commas) are located at the strict twofold axis of the icosahedral structure (23). (B) The predicted T=1 structure of the 23-nm VLPs would be formed by 60 units of the capsid protein arranged as 30 dimers (solid commas) surrounding the fivefold axis. (Modified from an article by Harrison [15], with permission of Elsevier Science Ltd.)

protein of NV, a calicivirus with T=3 icosahedral symmetry, has the structural flexibility to assemble into an alternate structure in the absence of RNA. This alternate structure of 23 nm in diameter exhibits antigenic domains, patterns of protection to trypsin cleavage, and cell binding sites similar to those of the T=3 particle; it is formed predominantly by full-length capsid protein that assembles through oligomeric intermediates that are common to the in vitro assembly pathway of particles of both sizes. Based on our data, we predict that the capsid protein in both types of VLPs folds into the same domains and that the different structures result from different subunit interactions during assembly. The observed size of the small particles corresponds to that expected for a T=1 structure formed by a capsid protein of 58 kDa that forms a T=3 structure of 38 nm. We predict that the 23-nm VLPs would be formed when 60 units of the capsid protein assembles into a structure with T=1 symmetry (Fig. 4). We propose that dimers of the capsid protein are common intermediates in the assembly of both types of particles and that during assembly the dimers adopt different orientations that result in different structures. Mass density calculations and the location and shape of the capsomers indicate that these represent dimers of the capsid protein, suggesting that preformed dimers might be the building blocks in the assembly of the rNV VLPs (24).

The three-dimensional structure of the rNV VLPs shows two types of capsomers or dimers based on their locations with respect to the icosahedral axes of symmetry. Sixty A dimers surround the icosahedral fivefold axes, and 30 B dimers are located at the strict twofold axes of the icosahedral structure (Fig. 4A). The two sides of a B dimer are equivalent as they are related to one another by the strict twofold axes, while the two sides of the type A arch are not strictly equivalent since they are related by the local twofold axes. This is also true for other spherical RNA plant viruses (tomato bushy stunt virus and SBMV) whose structures are known at atomic resolution (reviewed in reference 16). In these T=3 plant viruses, the N-terminal arm sequence is ordered only in the 30 B dimers, which create a flatter surface than the A dimers (16). In the predicted T=1 structure of the 23-nm VLPs, all the dimers would be equivalent to each other and would be located at the icosahedral twofold axis, surrounding the fivefold axis (Fig. 4B).

The 58K protein in the 23-nm VLPs undergoes N-terminal cleavage upon storage (Fig. 2B) more readily than the 38-nm VLPs. This different susceptibility to cleavage may be the result of the different organization of the N-terminal residues in the 23-nm VLPs compared to that in the T=3 38-nm VLPs. According to our hypothesis, all the monomers constituting the dimers in the 23-nm VLPs are equivalent, and the N-terminal residues would be equally susceptible to degradation. Our ob-

servations would be explained if the N-terminal residues within the B dimers in the 23-nm particles are more susceptible to degradation than the N-terminal residues in the A dimers that are present in the 38-nm VLPs but absent in the smaller structures. The higher susceptibility of the 58K protein in the 23-nm VLPs does not seem to be associated with instability of the particles, since particles with the cleaved pattern (Fig. 2B) are stable at 4°C for several months.

It has been shown that the N-terminal arm of the capsid proteins of SBMV, alfalfa mosaic virus, and brome mosaic virus is not necessary for the assembly of T=1 icosahedral particles when these residues are removed by trypsin treatment (4, 9, 16, 25). These observations agree with our results and suggest that the residues in the N terminus of the NV capsid protein may not be essential for the assembly of T=1 structures. Experiments are in progress to analyze the ability of N-terminally truncated mutants of the capsid protein to assemble into T=1 and T=3 particles.

The mechanism that controls the ability of the dimers to assemble into larger or smaller structures is still unknown for NV. The structure of small icosahedral plant viruses seen at high resolution shows that the flexibility in those viruses is built into the subunit contacts (1, 6, 7, 15). In the case of SBMV, it has been reported that the formation of T=3 and T=1 particles at low ionic strength from isolated SBMV coat protein and RNA might be regulated by the charge configuration of carboxyl group clusters in the putative initiation complex, a 10-mer or pentamer of dimers, which is common in the morphogenesis of both structures. The charge configuration, which is modulated by pH, divalent cations, and the presence of the arm domain, regulates further subunit interactions and hence the mode of T=3 versus T=1 assembly (7, 25).

The occurrence of particles of two sizes (35 to 40 and 15 to 20 nm in diameter) with a similar morphology in stool samples from patients infected with a human calicivirus, the Otofuke agent (27), suggests that the dimorphism described here is not an artifact caused by the overexpression of the capsid protein in insect cells.

Viral particles of two sizes (25 to 27 and 32 to 40 nm in diameter) have also been found in liver homogenates of rabbits and hares infected with one of two animal caliciviruses, rabbit hemorrhagic disease virus (RHDV) and European brown hare syndrome virus (3). These caliciviruses are responsible for an acute and fatal hepatitis of adult rabbits. However, the smaller particles, or core-like particles (CLPs), are composed of a 30K protein that has been reported to represent the N terminus of the capsid protein (2, 3, 10). These particles appear smooth by negative-stain electron microscopy and show different antigenic properties relative to the RHDV virions by immunoblotting and immunoelectron microscopy (10). The RHDV virions with a diameter of 32 to 40 nm are formed by the full-length capsid protein (VP60) and have typical calicivirus morphology with cup-like depressions. The characteristics of the small CLPs suggest that they are formed by a different mechanism than that for the 23-nm rNV VLPs described in this paper. The RHDV CLPs may be formed after proteolytic cleavage of the RHDV virions (3) or by expression of a truncated capsid protein (10), resulting in smooth, archless structures. It is likely that in these smaller structures for RHDV, the absence of the C-terminal domain of the capsid protein, and hence the absence of arch-like structures, accounts for the smaller diameter.

The in vivo significance of the assembly of smaller NV particles in the intestine in infected individuals is not clear. Now that it is clear that the NV capsid protein can form VLPs of two sizes, it will be of interest to see if such particles are detected and reported more frequently in clinical samples. We speculate that the smaller particles may originate in the intestinal lumen, which may provide the conditions for the reassembly of the smaller forms from alkaline-dissociated particles. These particles are likely noninfectious since they appear empty in negative-stain electron microscopy and size restrictions of these smaller particles may not permit encapsidation of a full-length genome. The ratio of smaller to typical-size particles will determine the particle/PFU ratio and may affect the infectivity of the virus; this may vary among individuals depending on differences in local conditions along the intestine. In the livers of rabbits infected with the calicivirus RHDV, the proportion of the small particles (CLPs) to the typical calicivirus particles has been associated with the course of the disease and the age of the rabbits. In acute rabbit hemorrhagic disease, few CLPs have been detected relative to the high number of typical calicivirus particles. On the other hand, protracted disease has been associated in young rabbits with the production of CLPs only (10). It will be of interest to determine whether the presence and relative proportion of smaller NV particles in infected individuals have any impact on the outcome of the disease.

The in vitro disassembly-reassembly system developed will be used to address how factors such as pH, divalent-cation concentration, and ionic strength affect the assembly of one structure versus the other. Additionally, electron cryomicroscopy techniques will be used to determine the triangulation number and other structural properties of these particles. The biochemical and structural characterization of assemblies such as 23-nm rNV VLPs will contribute to our understanding of the protein interactions that govern the assembly of small icosahedral viral capsids, as well as the factors that control the structural flexibility during assembly in vivo.

The potential uses of rNV VLPs as carriers to deliver heterologous epitopes to the mucosal sites of the immune system, or as intracellular carriers of heterologous genes, will be aided by a thorough understanding of the mechanisms by which the NV capsid protein assembles into different structures and by the identification of the domains involved in the protein-protein interactions in morphogenesis of these capsids.

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Homotypic and Heterotypic IgG and IgM Antibody Responses in Adults Infected With Small Round Structured Viruses

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Antibody responses to recombinant Norwalk (rNV) and Mexico (rMXV) viral capsid proteins were studied in 39 adults involved in outbreaks of gastroenteritis associated with genogroup 2 small round structured viruses (SRSVs). Nineteen individuals were involved in outbreaks associated with MXV-like strains and 20 in outbreaks associated with four other genogroup 2 SRSVs. IgG antibodies were measured in acute and convalescent sera using indirect enzyme-linked immunosorbent assay (ELISA), and IgM was measured by indirect and capture ELISAs. Nineteen (49%) patients demonstrated a significant rise in IgG to rMXV with four (10%) patients also showing anamnestic responses to rNV. Fourteen patients were positive in the rMXV IgM-capture ELISA, representing 74% of patients demonstrating IgG rises. IgG and IgM responses to rMXV were observed in both groups, although higher levels of responses were seen in adults infected with MXV-like strains than those infected with non-MXV genogroup 2 viruses. No significant IgM responses were observed to rNV. These results indicate that, following SRSV infection, adults show a rise in antibody which is broadly reactive to viruses within but not between genogroups, although greater homotypic than heterotypic responses are produced. These findings have implications for interpretation of seroepidemiological studies and serodiagnosis of SRSV infections using recombinant capsids. *J. Med. Virol. 54:305–312, 1998.*

KEY WORDS: human calicivirus; Norwalk virus; Mexico virus; ELISA; RT-PCR

INTRODUCTION

Small round structured viruses (SRSV) or Norwalk-like viruses are an antigenically diverse group of viruses which are now known to be caliciviruses. These agents cause outbreaks and sporadic cases of gastroenteritis with a particular predilection for producing illness in adults. Diagnosis has largely relied on the demonstration of characteristic virus-like particles in acute faecal samples by electron microscopy (EM) although the demonstration of a fourfold or greater rise in IgG to Norwalk virus between acute and convalescent serum samples has also been used to implicate SRSVs in the aetiology of outbreaks of gastroenteritis [Green et al., 1993].

Reverse transcription polymerase chain reaction (RT-PCR) has been developed for identifying SRSV RNA in faecal samples [Ando et al., 1995; Green et al., 1995b; Jiang et al., 1992a]. This technique has been used for both diagnosis and genetic comparison of antigenically distinct viruses as determined by solid phase EM (SPIEM) [Ando et al., 1994; Norcott et al., 1994]. Such phylogenetic analyses reveal considerable genetic variation within two distinct genogroups [Moe et al., 1994]: Genogroup 1 includes NV, Southampton (SOT), and Desert Shield (DSV) viruses; Genogroup 2 contains Bristol (BRV), Hawaii (HV), MXV, Snow Mountain (SMA), and Melksham (MKV) viruses [Lew et al., 1994a; Green et al., 1994, 1995a].

Radioimmunoassays, blocking enzyme immunoassays (EIA), and biotin-avidin EIAs have been described for the detection of IgG, IgA, and IgM antibodies based on reagents obtained from volunteers challenged with SRSVs [Blacklow et al., 1979; Dolin et al., 1986; Erdman et al., 1989; Greenberg et al., 1978; Madore et al., 1986; Treanor et al., 1988]. More recently, the expres-

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sion of the capsid region of cloned SRSVs in the baculovirus system resulting in the production of virus-like particles has revolutionised the production of high-quality antigen, allowing further development of serological assays [Jiang et al., 1992b]. NV was the first SRSV to be expressed in baculovirus, but recently recombinant capsids have also been produced for DSV, MXV, BRV, and HV [Dingle et al., 1995; Jiang et al., 1995; Lew et al., 1994b; Green et al., 1997].

Studies of serological responses to SRSVs using recombinant capsids indicate that infections result in a rise in serum IgG and IgA antibodies with IgM being observed in the majority of volunteers [Gray et al., 1994; Treanor et al., 1993]. Typically, IgM develops about 1 week following illness [Erdman et al., 1989]. Most symptomatic volunteers have pre-existing IgG antibody, but this does not correlate with susceptibility to infection [Graham et al., 1994]. Resistance to rechallenge with NV has been demonstrated in volunteers, but immunity appears to be short-lived [Wyatt et al., 1974]. Serological assays based on recombinant NV appear more sensitive and specific than those based on human material [Green et al., 1993]. In addition, differences in sensitivities of both IgG and IgM assays are observed between volunteers and patients involved in field outbreaks [Erdman et al., 1989]. Although this phenomenon was observed using assays based on faecal-derived antigen, which may not be the case with recombinant protein, it likely reflects antigenic differences between the infecting strain and the SRSV used in the assay. Homotypic responses are being measured in volunteers, whereas in field outbreaks heterotypic responses are largely being detected.

A number of studies have investigated homotypic and heterotypic responses to SRSVs. Madore and colleagues compared reagents obtained from volunteers challenged with NV, SMA, and HV and demonstrated cross-reactive responses between HV and SMA but less cross-reactivity between these strains and NV [Madore et al., 1990]. Parker studied heterotypic and homotypic SRSV responses in volunteers challenged with NV and in patients involved in SRSV-associated outbreaks using rNV and rMXV capsids [Parker et al., 1995]. Anamnestic responses to rMXV were observed in a proportion of the volunteers demonstrating good responses to rNV and vice versa in patients involved in outbreaks associated with SMA-like viruses. However, some inconsistencies were observed, with patients involved in outbreaks associated with HV-like strains failing to produce rises to rMXV.

Interpretation of studies on serological responses to SRSVs in patients involved in field outbreaks has been hindered by uncertainty in the identity of the infecting SRSV. Indeed, dual infections with distinct SRSVs have been described, which may result in further confusion [Kohn et al., 1995]. The purpose of this study was to investigate homotypic and heterotypic responses to infection with SRSVs in patients involved in outbreaks where the infecting SRSV was available for genetic characterisation. Thus, we were able to compare a panel of paired sera from outbreaks associated with MXV-like viruses with a similarly sized panel of serum samples from patients involved with outbreaks associated with SRSVs genotypically distinct from MXV but belonging to the same genogroup. IgG and IgM responses were measured using assays based on rMXV and rNV capsid proteins.

MATERIALS AND METHODS

Patients

Acute and convalescent serum specimens were retrospectively sought from symptomatic adults involved in outbreaks of gastroenteritis associated with SRSVs. Cases were enrolled into the study where paired sera were received at least 1 week apart, and sufficient SRSV positive faecal material was available from the outbreak to allow RT-PCR and sequence analysis. Cases were recruited from hospital outbreaks and incidents occurring in residential homes for the elderly and included both patients and staff. Nineteen sera were available from three outbreaks of gastroenteritis (RBH/93/UK, AGH/94/UK, and RBH/94/UK) which occurred in the winter of 1993/94 during an epidemic of MXV [Lewis et al., 1997]. Twenty paired sera were also available from cases involved in eight outbreaks of diarrhoea and vomiting which took place between 1990 and 1996.

Genetic Characterisation of SRSV

RT-PCR and amplicon sequencing were performed on SRSV-positive faecal specimens using the primer pair 36/E3 using methods described previously [Hale et al., 1996]. For those strains failing to amplify with 36/E3 the primer pair G2/E3 was used [Gray et al., 1997]. Thus, sequences were available from at least a 231-nucleotide region encoding part of the putative RNA polymerase to allow comparison to published SRSV sequences.

MXV and NV Indirect-IgG and IgM ELISAs

Indirect ELISAs were performed in 96-well U-bottomed microtitre plates (Nunc Immuno Maxisorp, 449824) using methods described by Gray, with some modifications [Gray et al., 1994]. Recombinant capsids were stored at −20°C in aliquots at a concentration of 2 mg/ml prior to use. Recombinant MXV capsids were used to coat plates at a dilution of 1:2,000 in phosphate-buffered saline (PBS; pH 7.4) and rNV capsid protein at a dilution of 1:3,000. All further dilutions were made in PBS containing 1% skim milk. For the IgG assays, doubling serum dilutions were made from 1:100, and a 1:3,000 peroxidase conjugated goat anti-human IgG (Sigma-Aldrich Company Ltd., Poole, Dorset, UK) was used as conjugate. In the IgM assays sera were pretreated to remove IgG and IgA using goat anti-human IgA (Sigma) and goat anti-human IgG (Sigma) both at a ratio of 1:20 (human sera:goat sera). Samples were absorbed by reaction overnight at 4°C and tested at a final dilution of 1:100. A 1:2,000 dilution of peroxidase-conjugated goat anti-human IgM (Sigma) was used in

the final stage of the IgM assays. In all assays, 100 µl of TMB (Europa Research Products, Cambridge, UK) was used as substrate with the reaction being stopped after 20 min by the addition of 100 µl of 1 M sulphuric acid (H_2SO_4). Well optical densities (OD) were measured using the A450/A620 dual wavelength mode of an enzyme-linked immunosorbent assay (ELISA) reader (MCC340 MkII, Wellcome Diagnostics).

MXV and NV capture-IgM ELISAs

ELISA plates were coated with 100 µl of goat anti-human IgM (Tago-immunologicals, Bio-source International, Camarillo, CA) at a dilution of 1:3,000 in 0.01 M carbonate coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$; pH 9.6) and incubated for 4 hr at room temperature. Plates were washed once with PBS containing 0.05% Tween (PBST), blocked by the addition of 200 µl of PBS containing 5% skim milk, and then left overnight at 4°C. Following two washes with PBST, 100 µl of acute and convalescent serum samples diluted at 1:100 in PBS/1% skim milk were added, and the plates were incubated at 37°C for 2 hr. Plates were washed six times, and 100 µl of either rNV or rMXV was added at the same concentrations as used in the indirect ELISAs. Plates were again left overnight at 4°C and washed a further six times. Either 100 µl of 1:10,000 dilution of guinea pig anti-rMXV antibody (MXV assay) or 1:160,000 dilution of rabbit anti-rNV (NV assay) was added, and the plates were incubated at 37°C for 2 hr. Following six washes with PBST, 100 µl of 1:3,000 dilution of peroxidase-conjugated goat anti-guinea pig IgG (Sigma), for the MXV assay, or 100 µl of 1:3,000 dilution of peroxidase-conjugated goat anti-rabbit IgG (Sigma), for the NV assay, both containing 1% normal human serum, were added and incubated for 2 hr at 37°C. After a final six washes, 100 µl of TMB substrate was added to each well, and the reaction stopped and the plates were read as above.

Optimal dilutions of recombinant capsids, hyperimmune animal antisera, and conjugated antibodies were determined prior to testing. Inclusion of 1% normal human sera with the conjugate was found to significantly reduce background in negative control wells, although use of normal goat serum at other stages did not appear to significantly improve the performance of the assays. Duplicate negative and positive controls were included in each plate, which were derived from acute and convalescent sera from a symptomatic volunteer challenged with NV and an adult involved in an outbreak associated with MXV.

Interpretation of ELISAs

Titres in the rMXV and rNV IgG-indirect ELISAs were defined as the highest dilution giving an OD > 0.1. In the indirect and capture-IgM ELISAs convalescent sera were arbitrarily considered positive if OD > 0.1 and the ratio of the OD in the convalescent sera to that of the acute sera (P/N) was >2. This cut-off was adopted as a number of sera showed non-specific reactivity. For samples giving P/N values >2 in the IgM-capture assay, reactivity was shown to be specific as preabsorption with goat anti-human IgM removed or significantly reduced ODs in the ELISA, whereas identical treatment with normal goat serum did not (data not shown).

RESULTS

Patient Demographics

The sex distribution was similar for the two study groups: ten females and seven males for the MXV-like infected group and eight females and four males for the group infected with non–MXV-like strains (information was not available on remaining cases). The MXV-infected group was aged between 26 and 99 with a mean of 74 years, and for the 15 individuals in which age was stated in the non–MXV-like infected group, the age range was 23–91 years with a mean of 56. The difference in age between the two groups was significant ($P = .04$, Student's t-test).

Characterisation of SRSV Strains

Faecal specimens from three outbreaks (RBH/93/UK, AGH/94/UK, and RBH/94/UK) were highly reactive in the rMXV antigen ELISA identifying these as MXV-like strains [Hale et al., 1996; Lewis et al., 1997]. The sequence of AGH/94/UK was available from one of these previous studies [Hale et al., 1996]. Faecal samples from the eight remaining outbreaks were subject to RT-PCR and amplicon sequencing, and the amino acid alignment for this region and the percentage similarity to MXV at the nucleotide level is shown in Figure 1. Phylogenetic analysis revealed that strains clustered into four groups: Strains from two outbreaks (CA/92/UK and SJH/92/UK) were BRV-like [Green et al., 1994], one outbreak (MH/90/UK) was caused by a MKV-like strain [Green et al., 1995a], strains from two outbreaks (Sea/91/UK and WGH/93/UK) were HV-like [Lew et al., 1994a,b], and strains from the remaining three outbreaks (ELH/96, CO/96 and RHCH/96) appeared unique. These strains were most closely related to BRV, exhibiting 92% sequence similarity, but had three amino acid substitutions over this region of ORF 1 (Fig. 1). This strain was first seen associated with an outbreak of gastroenteritis which occurred in Grimsby in 1995 (J. Green, personal communication), and hence, these are referred to as Grimsby-like viruses (GRV). The precise genetic and antigenic relationship of GRV to BRV has yet to be determined. These four genetic clusters align within SRSV genogroup 2, displaying a 70–73% similarity to MXV over a 231-base region of the RNA polymerase gene.

Determination of IgG Responses Using rMXV and rNV Capsids

Results of indirect-IgG ELISAs using MXV and NV recombinant capsids and are shown in Tables I and II. Nineteen (49%) of 39 patients had a fourfold or greater rise in IgG antibody to rMXV. Ten of 19 (53%) were infected with MXV-like viruses (Table I), while nine of 20 (45%) were associated with other SRSV genogroup 2

	10															20															30															40
Mexico virus	R	F	S	A	E	P	Q	L	A	Q	I	V	A	E	D	L	L	A	P	S	V	V	D	V	G	D	F	K	I	T	I	N	E	G	L	P	S	G	V	P
AGH/94/UK/MXV	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Melksham virus	K	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	S	.	.	.	.	.	.	.	.	.	.	.	S	.	.	.	.	.	.	.	.	.	.
MH/90/UK/MKV	K	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	S	.	.	.	.	.	.	.	.	.	.	.	S	.	.	.	.	.	.	.	.	.	.
Hawaii virus	K	.	.	P	.	.	H	.	.	.	V	.	.	.	.	.	.	.	S	.	.	.	M	.	.	.	.	.	.	.	S	.	.	.	.	.	.	.	.	.	.
Sea/91/UK/HV	K	.	.	P	.	.	H	.	.	.	V	.	.	.	.	.	.	.	S	.	.	.	M	.	.	.	.	.	.	.	S	.	.	.	.	.	.	.	.	.	.
WGH/93/UK/HV	K	.	.	P	.	.	H	.	.	.	V	.	.	.	.	.	.	.	S	.	.	.	M	.	.	.	.	.	.	.	S	.	.	.	.	.	.	.	.	.	.
Bristol virus	K	.	.	P	.	.	H	.	.	.	.	.	.	.	.	.	.	.	S	.	.	.	M	.	.	.	.	.	.	.	S	.	.	.	.	.	.	.	.	.	.
CA/92/UK/BRV	K	.	.	P	.	.	H	.	.	.	.	.	.	.	.	.	.	.	S	.	.	.	M	.	.	.	.	.	.	.	S	.	.	.	.	.	.	.	.	.	.
SJH/92/UK/BRV	K	.	.	P	.	.	H	.	.	.	.	.	.	.	.	.	.	.	S	.	.	.	M	.	.	.	.	.	.	.	S	.	.	.	.	.	.	.	.	.	.
ELH/96/UK/GRV	K	.	.	S	.	.	H	.	.	.	V	.	.	.	.	.	.	.	S	.	.	.	.	.	.	.	.	.	.	.	S	.	.	.	.	.	.	.	.	.	.
RHCH/96/UK/GRV	K	.	.	S	.	.	H	.	.	.	V	.	.	.	.	.	.	.	S	.	.	.	.	.	.	.	.	.	.	.	S	.	.	.	.	.	.	.	.	.	.
CO/96/UK/GRV	K	.	.	S	.	.	H	.	.	.	V	.	.	.	.	.	.	.	S	.	.	.	.	.	.	.	.	.	.	.	S	.	.	.	.	.	.	.	.	.	.

	50															60															70															% Similarity to MXV
Mexico virus	C	T	S	Q	W	N	S	I	A	H	W	L	L	T	L	C	A	L	S	E	V	T	G	L	G	P	I	I	Q	A	N	S	M	Y	S	F							
AGH/94/UK/MXV	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.							98
Melksham virus	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	D	.	S	.	.	V	.	.	.	.	L	F	.	.							71
MH/90/UK/MKV	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	D	.	S	.	.	V	.	.	.	.	L	F	.	.							73
Hawaii virus	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	D	.	S	.	.	.	.	.	.	.	L	F	.	.							71
Sea/91/UK/HV	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	I	.	.	D	.	S	.	.	.	.	.	.	.	L	F	.	.							70
WGH/93/UK/HV	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	D	.	S	.	.	.	.	.	.	.	L	F	.	.							71
Bristol virus	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	D	.	S	.	.	.	.	.	.	.	L	F	.	.							73
CA/92/UK/BRV	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	D	.	S	.	.	.	.	.	.	.	L	F	.	.							73
SJH/92/UK/BRV	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	D	.	S	.	.	.	.	.	.	.	L	F	.	.							73
ELH/96/UK/GRV	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	D	.	S	.	.	.	.	.	.	.	L	F	.	.							71
RHCH/96/UK/GRV	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	D	.	S	.	.	.	.	.	.	.	L	F	.	.							71
CO/96/UK/GRV	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	D	.	S	.	.	.	.	.	.	.	L	F	.	.							71

Fig. 1. Amino acid alignment of SRSVs characterised by RT-PCR and direct sequencing of a portion of ORF1 corresponds to locations 4634 to 4864 of NV. Strains are grouped by sequence similarity to previously characterised SRSVs (in bold), and virus notation indicates related prototype. ELH/96/UK, RHCH/96/UK, and CO/96/UK appear to be sufficiently divergent from BRV to classify as a unique genetic cluster (referred to as GRV-like). Percentage nucleotide similarity to MXV is shown.

viruses (Table II). Of these 19 individuals four also showed a fourfold rise in IgG to rNV. IgG rises to rMXV were seen in patients involved in outbreaks associated with strains from all non-MXV genetic clusters. The geometric and arithmetic mean values of acute and convalescent IgG titres to rMXV and rNV were calculated for the MXV-infected group and those infected with other SRSV genogroup 2 strains and compared using the *t*-test (Table III).

Measurement of IgM by Indirect and Capture ELISAs Based on rMXV and rNV

Seven (18%) of 39 patients were positive in the rMXV indirect-IgM ELISA of whom six were infected with MXV-like strains and one was infected with a non-MXV genogroup 2 virus. Six of these seven patients demonstrated a significant rise in IgG titre to rMXV. Fourteen (36%) patients were positive in the rMXV capture-IgM ELISA, which includes all but one patient positive in the rMXV indirect IgM assay. All patients positive by capture-IgM had a fourfold or greater rise in IgG titre to rMXV. Eight patients were infected with MXV-like strains and six with non-MXV genogroup 2 viruses. The mean OD of the convalescent serum of the MXV-infected patients positive in the IgM capture assay was 0.889 compared to 0.265 for the group infected with non-MXV SRSV genogroup 2 viruses.

One patient (15B) was positive in both rNV IgM ELISAs and also in the rMXV indirect-IgM ELISA. However, there was no corresponding rise in IgG to either antigen, suggesting that these were false-positive results. Insufficient sera were available to test for rheumatoid factor or to repeat the indirect-IgM tests following further IgG and IgA absorption. All other specimens tested were negative in the rNV indirect-IgM and capture-IgM ELISAs.

DISCUSSION

Serological assays based on NV have been extensively used for the diagnosis of SRSV-associated outbreaks and for epidemiological studies. However, interpretation of these data has been difficult because of a lack of understanding of homotypic and heterotypic humoral responses to this antigenically diverse group of viruses. The recent cloning and expression of MXV capsid now provides reagents based on a strain which is antigenically and genetically distinct from NV, thus permitting a more thorough investigation of antibody responses following infection with SRSVs. In addition, genogroup 2 SRSVs have been more prevalent in the United Kingdom in recent years.

In this study we compared seroresponses of patients involved in outbreaks of gastroenteritis associated with MXV and patients involved in outbreaks associated with non-MXV genogroup 2 viruses. Fifty percent of patients showed a fourfold or greater rise in IgG to rMXV, and 10% exhibited anamnestic responses to rNV [Parker et al., 1995]. Gray and colleagues demonstrated IgG rises to rNV in all volunteers with symptomatic NV infection [Gray et al., 1993]. The discrep-

TABLE I. Results of 19 Serum Pairs From Patients Involved in Outbreaks of Gastroenteritis Associated With MXV-Like Strains Tested in Indirect and Capture ELISAs Based on Recombinant MXV and NV Capsids*

Outbreak	Case ID	Day[a]	MXV IgG	MXV Cap-IgM	MXV Ind-IgM	NV IgG
AGH/94/UK/MXV (A)	1A	3	3,200	Pos	Neg	12,800
		23	12,800	P/N = 2		25,600
	2A	3	51,200	Neg	Neg	6,400
		23	51,200			3,200
	3A	3	1,600	Neg	Neg	6,400
		23	1,600			6,400
	4A	4	3,200	Pos	Neg	12,800
		24	102,400	P/N = 2.9		25,600
	5A	6	204,800	Neg	Neg	6,400
		23	102,400			6,400
	6A	3	100	Neg	Neg	3,200
		20	25,600			6,400
	7A	2	1,600	POS	POS	100
		28	102,400	P/N = 12	P/N = 23	400
	8A	4	3,200	Neg	Neg	1,600
		33	3,200			3,200
	9A	2	3,200	Neg	Neg	1,600
		30	3,200			1,600
RBH/93/UK/MXV (B)	10B	NK	6,400	POS	Neg	6,400
			102,400	P/N = 3		6,400
	11B	NK	12,800	POS	Pos	6,400
			819,200	P/N = 7	P/N = 2.2	12,800
	12B	NK	1,600	POS	Neg	1,600
			204,800	P/N = 4.9		3,200
	13B	NK	25,600	POS	Pos	6,400
			819,200	P/N = 14	P/N = 3	12,800
	14B	NK	3,200	POS	POS	6,400
			409,600	P/N = 24	P/N = 44	6,400
	15B[b]	3	204,800	Neg	POS	25,600
		11	102,400		P/N = 6	6,400
RBH/94/UK/MXV (C)	16C	7	102,400	Neg	Neg	12,800
		21	51,200			6,400
	17C	7	409,600	Neg	Neg	12,800
		21	409,600			12,800
	18C	7	102,400	POS[c]	POS	400
		21	1,638,400	P/N = 4	P/N = 18	800
	19C	7	6,400	Neg	Neg	51,200
		21	12,800			102,400

*IgG rises ≥4 are indicated by underlining, and positive IgM values with P/N values between 2 and 3 are indicated by lower case (see Materials and Methods for derivation of P/N). NK, not known.
[a]Number of days after onset of symptoms.
[b]Also positive in NV Cap-IgM and NV Ind-IgM ELISAs.
[c]IgM was present in first serum (OD = 0.7 and rose to 3.0 by day 21).

ancy between their observation and ours deserves further comment. First, faecal specimens were not available from all cases included in this study, and thus, it is possible that a proportion of patients had diarrhoea due to some other cause. Second, there was variability in the timing of collection of acute specimens, and patients may have already been mounting an immune response. This is plausible, as the mean time of collection of acute specimens of cases demonstrating a rise in IgG was 2.3 days compared to 5 days for those failing to show a rise. This difference is significant ($P < .005$, t-test). Finally, many patients in this study were elderly, and their immune responses may not be comparable to those in volunteers who were generally younger.

The proportion of cases showing a significant rise in IgG to rMXV was similar for both the group infected with MXV-like viruses and non-MXV genogroup 2 viruses. Thus, significant heterotypic responses were observed between SRSV strains within genogroup 2. IgG titres to rMXV were significantly higher in the second sera of individuals infected with MXV-like strains that non-MXV genogroup 2 strains. There was also a smaller but significant difference in IgG titres to rMXV in the first serum sample (Table III). However, despite this, the MXV-infected group still had a significantly greater increase in serum IgG titre to rMXV than patients infected with non-MXV genogroup 2 SRSVs (mean rise of 38-fold vs. eightfold, $P = .05$, t-test), indicating greater homotypic than heterotypic IgG responses. The difference in IgG titres to rMXV in the first sera between the two groups may either reflect the lateness in collection of acute samples or the older age of the MXV-infected group. No difference was observed in IgG titres to rNV (Table III), supporting the former hypothesis.

IgM responses were only observed in 18% of the total study group using the rMXV indirect ELISA. The majority of patients had an accompanying rise in IgG titre to rMXV. The individual who failed to show a rise in

TABLE II. Results of 20 Serum Pairs From Patients Involved in Outbreaks of Gastroenteritis Associated With Non-MXV Genogroup 2 SRSVs Tested in Indirect and Capture ELISAs Based on Recombinant MXV and NV Capsids*

Outbreak	Case ID	Day[a]	MXV IgG	MXV Cap-IgM	MXV Ind-IgM	NV IgG
CA/92/UK/BRV	20D	2	800	Neg	Neg	6,400
(D)		9	25,600			25,600
SJH/92/UK/BRV	21E	3	6,400	Neg	Neg	12,800
(E)		12	6,400			12,800
	22E	3	400	Neg	Neg	400
		12	200			200
	23E	3	6,400	Neg	Neg	1,600
		12	6,400			1,600
Sea/91/UK/HV	24F	1	800	Neg	Neg	1,600
(F)		37	12,800			6,400
WGH/93/UK/HV	25G	2	3,200	Neg	Neg	25,600
(G)		18	3,200			51,200
	26G	3	1,600	POS	POS	800
		20	51,200	P/N = 7.5	P/N = 10	1,600
MH/90/UK/MKV	27H	2	6,400	Pos	Neg	6,400
(H)		12	25,600	P/N = 2.9		6,400
	28H	2	3,200	Pos	Neg	400
		12	25,600	P/N = 2.6		400
	29H	11	6,400	Neg	Neg	6,400
		23	6,400			12,800
ELH/96/UK/GRV	30I	7	6,400	Neg	Neg	6,400
(I)		35	6,400			3,200
	31I	5	12,800	Neg	Neg	12,800
		31	25,600			12,800
	32I	5	12,800	Neg	Neg	25,600
		31	25,600			25,600
	33I	11	6,400	Neg	Neg	25,600
		35	12,800			51,200
RHCH/96/UK/GRV	34J	4	3,200	Neg	Neg	400
(J)		39	6,400			800
	35J	1	800	Pos	Neg	12,800
		44	25,600	P/N = 2.5		12,800
CO/96/UK/GRV	36K	4	6,400	Neg	Neg	12,800
(K)		32	6,400			12,800
	37K	5	3,200	Neg	Neg	12,800
		33	25,600			25,600
	38K	1	3,200	Pos	Neg	800
		29	12,800	P/N = 3		3,200
	39K	1	1,600	Neg	Neg	12,800
		29	6,400			12,800

*IgG rises ≥4 are indicated by underlining, and positive IgM values with P/N values between 2 and 3 are indicated by lower case (see Materials and Methods for derivation of P/N).
[a]Number of days after onset of symptoms.

IgG was also positive in the rNV indirect-IgM ELISA, and this probably represents a false-positive, possibly due to incomplete absorption of IgG and IgA. Unfortunately insufficient sera were available to repeat the absorption step. The relatively low sensitivity of the indirect-IgM assay led us to develop the capture-IgM ELISA, and our results show that this assay detected IgM responses in a greater proportion of SRSV infections; 35% of all patients were positive in the assay based on rMXV. This represents 74% of those demonstrating a rise in IgG to rMXV. The IgM responses observed, however, were relatively poor, with seven of the 13 positives giving P/N values between 2 and 3 (Tables I, II). Low level IgM responses following SRSV infections have also been observed by others and may reflect reinfections or the mucosal nature of the infection [Erdman et al., 1989; Gray et al., 1994; Johnson et al., 1990].

Similar to IgG responses the IgM assays also suggest a greater homotypic response. Only one individual was positive in the indirect IgM assay from the group infected with non-MXV strains in comparison to six of 19 infected with MXV-like strains. In the rMXV capture-IgM ELISA the rate of detection was 25% (5 of 20) in individuals infected with non-MXV genogroup 2 viruses and 47% (9 of 19) with MXV, with generally higher ODs observed in the second sera of the MXV-infected group (although this did not reach statistical significance). The observation of broadly reactive genogroup-specific seroresponses in adults infected with SRSVs supports the classification of SRSVs into two genogroups. Two hypotheses could explain this finding; that the immune response is directed at genogroup-specific epitopes as well as type-specific epitopes; alternatively, infection results in antibody production to SRSVs (within the same genogroup) previously encountered by the individual. This phenomenon is advantageous for serological diagnosis of SRSV infec-

TABLE III. IgG Titres in Acute and Convalescent Sera to rMXV and rNV in Patients Infected With MXV-Like and Non-MXV Genogroup Strains*

	Patients infected with MXV-like strains			Patients infected with other SRSV G2 strains			Difference in arithmetic means and significance[a]
	No. of sera	Arithmetic mean of log titres ± SD	Geometric mean	No. of sera	Arithmetic mean of log titres ± SD	Geometric mean	
rMXV antigen							
First serum	19	4.012 ± 0.94	10,280	20	3.505 ± 0.43	4,620	0.51 (P = .04)
Second serum	19	4.836 ± 0.86	68,550	20	4.017 ± 0.52	15,850	0.82 (P < .001)
Difference in arithmetic means and significance[b]		0.82 (P < .002)			0.51 (P < .001)		
rNV antigen							
First serum	19	3.679 ± 0.63	4,775	20	3.656 ± 0.64	4,530	0.02 (ns)
Second serum	19	3.806 ± 0.55	6,400	20	3.806 ± 0.68	6,400	0
Difference in arithmetic means and significance		0.13 (ns)			0.15 (P = .02)		

*P values determined by the t-test ([a]equal variances, [b]paired samples).
SD, standard deviation; ns, not significant.

tions, as our results would predict that IgG rises to either rMXV or rNV would be observed frequently following infection with either genogroup 2 or genogroup 1 SRSVs, respectively. Conversely, a rise in IgG to a recombinant SRSV capsid protein cannot be construed as evidence of recent infection with homotypic virus. Alternative methods will be required to measure type-specific responses to SRSVs, and peptide mapping is one approach which may be useful in identifying type-specific epitopes.

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A One-Tube Method of Reverse Transcription-PCR To Efficiently Amplify a 3-Kilobase Region from the RNA Polymerase Gene to the Poly(A) Tail of Small Round-Structured Viruses (Norwalk-Like Viruses)

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Amplification of a 3-kb genome region from the RNA polymerase gene to the 3′ poly(A) tail of small round-structured virus (SRSV) by reverse transcription-PCR (RT-PCR) has been difficult to achieve because of a stable secondary structure in a region between the RNA polymerase gene and the 5′ end of the second open reading frame. We have developed a one-tube RT-PCR method to efficiently amplify this region. The method comprises three procedures: purification of poly(A)$^+$ RNA from a starting RNA solution by oligo(dT)$_{30}$ covalently linked to latex particles, buffer exchange, and continuous RT and PCR in a single tube containing all reaction components. The key elements of this method are (i) first-strand cDNA synthesis with the Superscript II version of RNase H$^-$ Moloney murine leukemia virus reverse transcriptase at 50°C for 10 min by using the RNA-oligo(dT)$_{30}$ hybrid on the latex particles as the template and primer, and (ii) PCR by *Taq* and *Pwo* DNA polymerases mixed together with a mixture of 12 phased oligo(dT)$_{25}$ antisense primers. The detection threshold of the one-tube RT-PCR method was as little as 0.2 ng of the crude RNA used as the source of the template. Using this method, we obtained 3-kb products from 24 SRSV strains previously characterized into four genetic groups. These included 5 P1-A, 4 P1-B, 5 P2-A, and 10 P2-B strains. Because SRSVs have not yet been cultivated in vitro, this novel method should facilitate molecular characterization of SRSVs to provide a firm scientific foundation for improvements and refinements of SRSV diagnostics.

Small round-structured viruses (SRSVs), also called Norwalk-like viruses, are members of the family *Caliciviridae* which have a single-stranded RNA genome with positive polarity, are 7.6 to 7.7 kb in length, and have a poly(A) tail at the 3′ end (13, 17, 22, 24, 39). The genome contains three open reading frames (ORFs) and two small untranslated regions at the 5′ and 3′ termini. The largest ORF, ORF1, encodes a polyprotein precursor for nonstructural proteins that includes a putative RNA polymerase gene located toward the carboxyl terminus (17, 24, 29). The second ORF, ORF2, encodes the viral capsid protein, and the smallest ORF, ORF3, encodes a protein of undefined function (8, 15, 18, 21, 25–27). The viruses of this group have not yet been cultivated in vitro, no practical animal model has been developed to study them, and virus concentration in stool samples is usually very low.

SRSVs are the major cause of outbreaks of nonbacterial acute gastroenteritis and appear to be a common cause of sporadic episodes of gastroenteritis in children and adults (23, 35, 42). The genetic or pathogenetic relationships between the strains that cause outbreaks and those that simultaneously circulate in the same communities have not been studied, in part due to the lack of suitable methods to screen for SRSVs in large field studies. The reverse transcription-PCR (RT-PCR), currently used in many laboratories to detect and characterize SRSVs, is based on the nucleotide sequence of the RNA polymerase region that has the highest degree of nucleotide sequence conservation. While the RNA polymerase region may be suited to broadly detect SRSV strains, the ORF2 encoding the capsid protein is of particular interest because its sequence and expression provide clues to genetic and antigenic relationships among SRSVs that would be important for the development of improved diagnostic methods. As more information on the sequence from the RNA polymerase region has become available, many researchers have directed their efforts toward amplification of a region extending from the RNA polymerase gene to the poly(A) tail. The RT-PCR products obtained from the RNA polymerase region are small (100 to 500 bp) (3, 11, 12, 14, 20, 31, 33, 44), whereas the distance from this region to the poly(A) tail is about 3 kb and includes two stable secondary structures predicted in locations upstream from ORFs 2 and 3 (22). The RT-PCR protocols routinely used to amplify the RNA polymerase region have usually been inefficient for amplification of this region. To date, RT-PCR amplification of the 3-kb region of SRSVs has been reported for only three strains. Of these, Southampton virus was amplified by a protocol that required 0.5 g of a stool sample (24), an amount corresponding to 70 to 1,000 times more than that used for short products from the RNA polymerase region (3, 12, 14, 20). For the remaining two strains, Lordsdale and Mexico viruses, details of the amplification protocols have not been reported (13, 19).

We have systematically examined the RT-PCR conditions required to overcome the previous difficulties and have developed a one-tube RT-PCR method that permits routine amplification of the 3-kb region of genetically distinct SRSV strains present in low concentrations in stool samples. The availability of this novel RT-PCR method should facilitate molecular characterization of SRSVs. It may also be applicable with other single-stranded RNA viruses in which amplification of a long

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TABLE 1. SRSV strains used in this study

Strain code	Strain identification[a]	Sense primer(s) used
S1	P1-A/11523/93/La[b]	SR48
S2	P1-A/2036/92/US[c]	SR48 + SR50 + SR52
S3	P1-A/11873/94/Md	CL6 or SR48
S4	P1-A/8438/94/Ar	CL10
S5	P1-A/8548/94/Fl	CL14 or SR48
S6	P1-B/11551/93/Fl[d]	CL18
S7	P1-B/11821/93/Md[d]	CL18
S8	P1-B/11860/94/Pa	CL18
S9	P1-B/11641/94/Hi	CL18
S10	P2-A/2555/93/Pa	CL22
S11	P2-A/11885/94/La	CL22
S12	P2-A/8377/94/Al	CL22
S13	P2-A/8387/94/Md	CL22
S14	P2-A/11756/95/Vt	CL22
S15	P2-B/4880/86/US[c]	SR46
S16	P2-B/12700/92/UK[c]	SR46
S17	P2-B/12678/94/Md	CL26
S18	P2-B/12044/94/Va	CL26
S19	P2-B/8410/94/Hi	CL26
S20	P2-B/12630/94/US	CL26
S21	P2-B/12732/94/La	CL28
S22	P2-B/12275/94/Vt	CL28
S23	P2-B/11941/95/Fl	CL26
S24	P2-B/12096/95/Ak	CL26

[a] Probe group/strain designation/year/state or country of detection.
[b] The strain is identical to LA-Oyst-G1 described in the multistate oyster-associated outbreak (2).
[c] From Ando et al. (3).
[d] From Ando et al. (2).

region of the genome has been difficult because of its stable secondary structure or the small amount of the template RNA available.

MATERIALS AND METHODS

Virus samples. The 24 strains of SRSVs from outbreaks of acute gastroenteritis used in this study had previously been characterized into four genetic groups: P1-A, P1-B, P2-A, and P2-B (3). These strains are identified by probe group, specimen designation, year, and state or country of detection and were designated strains S1 to S24 for convenience in this study (Table 1). Strain S1 was used to optimize the one-tube RT-PCR conditions described below.

RNA. RNA was extracted from 100 μl of a 10% stool suspension as described previously (3), concentrated by ethanol precipitation, and suspended in 20 μl of H_2O. For strain S1, a 10% stool suspension was prepared from 1.7 g of a single stool sample and was kept at 4°C, and RNA was extracted several times in separate groups of 10 to 30 tubes during the development of the method. After each extraction, the RNA was pooled from the tubes, the RNA concentration was determined spectrophotometrically, and aliquots (2 to 10 μl/tube) were made for use in a single-tube per experiment. In the typical experiments performed to optimize the reaction conditions and determine the detection threshold of the method as described below (see Fig. 1 to 3), the RNA concentration was broadly estimated to be 10 ng per μl by spectrophotometry (optical densities at 260, 280, and 320 nm, 0.406, 0.326, and 0.177, respectively), but the concentration of the poly(A)$^+$ RNA was not determined. The RNA extracts from the 24 SRSV strains were kept at −70°C and used in the RT-PCR within 3 months.

RT-PCR primers. Nineteen primers were used in this study (Table 2). The oligo(dT)$_{30}$-Latex primer (Oligo-dT30 Super; Takahara Co. Ltd., Otsu, Japan) is an oligo(dT)$_{30}$ covalently linked at or near its 5′ end to the carboxyl residues on the surface of the latex particles. The VN primer is a mixture of 12 combinations of 40-mer oligonucleotides [linker-(dT)$_{25}$-VN, where V may be dG, dA, or dC and N may be any one of the four deoxynucleotides] in which the 12 components were synthesized and purified separately and then mixed to make a solution with an equal concentration of each component. The TT primer is a single 40-mer primer with the same sequence as the VN primer, except that two 3′ nucleotides, V and N, are substituted by two dTs. All but oligo(dT)$_{30}$-Latex primers were synthesized in the Biotechnology Core Facility of the Centers for Disease Control and Prevention (Atlanta, Ga.).

RT-PCR. Five microliters of the RNA solution was mixed with an equal volume of 10% (wt/vol) oligo(dT)$_{30}$-Latex suspension in a 0.5-ml microtube, heated at 90°C for 1 min, and rapidly cooled in ice-water. After 3 min in ice-water and the subsequent addition of 1.1 μl of 5 M NaCl (final 0.5 M), the tube was incubated at 37°C for 20 min and was then centrifuged at about 16,000 × *g* for 3 min at room temperature. The latex particles containing the RNA-oligo(dT)$_{30}$ hybrid were suspended in a total of 1 ml of washing buffer (10 mM Tris-HCl [pH 7.6 at room temperature], 0.5 M NaCl, and 1.0% [vol/vol] Triton X-100), transferred to a 1.5-ml microtube at room temperature, and centrifuged as described above. After being placed on ice, the latex particles were suspended in 1 ml of ice-cooled RT-PCR buffer (50 mM Tris-HCl [pH 8.7 at room temperature], 40 mM KCl, 2.5 mM $MgCl_2$, and 0.5% [vol/vol] Triton X-100), transferred to a fresh 1.5-ml tube, mildly pipetted on ice, and centrifuged at room temperature as described above. The latex particles were then suspended (on ice) in 400 μl of the ice-cooled RT-PCR reaction mixture (50 mM Tris-HCl [pH 8.7 at room temperature], 40 mM KCl, 2.5 mM $MgCl_2$, 0.5 mM [each] dATP, dGTP, dCTP, and dTTP [deoxynucleoside triphosphates; dNTPs], 2 mM [without accounting for carryover from the enzyme storage buffers] dithiothreitol, 0.5% [vol/vol] Triton X-100, 2% [vol/vol] [without accounting for the carryover] glycerol, 0.4 units of human placental RNase inhibitor [Boehringer Mannheim Corp.] per μl, 0.25 μM

TABLE 2. RT-PCR primers

No.	Primer	Length	Sequence (5′ to 3′)[a]	Polarity[b]	Location[c]
1	Oligo(dT)$_{30}$-Latex	30	Latex particle-(t)$_{30}$	−	
2	VN primer	40	gct gga gtc tag a (t)$_{25}$ (a/g/c)(a/g/c/t)	−	
3	TT primer	40	gct gga gtc tag a (t)$_{25}$ tt	−	
4	Mon189	25	gca cca att atg gct tgg gcc att a	−	6972
5	SR33	21	tgt cac gat ctc atc atc acc	−	4888
6	SR46	21	tgg aat tcc atc gcc cac tgg	+	4766
7	SR48	21	gtg aac agc ata aat cac tgg	+	4766
8	SR50	21	gtg aac agt ata aac cac tgg	+	4766
9	SR52	21	gtg aac agt ata aac cat tgg	+	4766
10	CL6	23	aac agc ata aat cac tgg **CTG AT**	+	4769
11	CL10	24	**ATC CTA ACT CTA TGT GCA TTG TCA**	+	4787
12	CL14	24	aac agc ata aat cac tgg **CTA AAT**	+	4769
13	CL18	24	gcc cac tgg **ATT CTA ACT TTG AGT**	+	4778
14	CL22	20	atc gcc cac tgg **TTG CTT AC**	+	4775
15	CL26	20	atc gcc cac tgg **CTC CTC AC**	+	4775
16	CL28	21	atc gcc cac tgg **CTT CTA ACT**	+	4775
17	SR34	20	tcg taa atg atg atg gcg tc	+	5352
18	SR62	23	tat cag (^t_c)t(^a_c) aag cct gtg gga ac	+	6888
19	SR60	20	tga g(^c_t)t c(^c_t)g gtg gct tca tg	+	7293

[a] The sequences specific for individual strains are highlighted by the boldface capital letters. VN primer, SR60, and SR62 are degenerate primers, with nucleotide variations indicated in parentheses.
[b] −, negative; +, positive.
[c] Equivalent location of the 5′ end of the primer within the unique genomic sequence (7,654 bases) of Norwalk virus (GenBank accession number, M87661).

[each] positive-sense primer(s) and VN primer, 0.05 U of *Taq* DNA polymerase [Boeringer Mannheim Corp.] per μl, 6.25×10^{-3} U of *Pwo* DNA polymerase [Boehringer Mannheim Corp.] per μl, and 3 U of the Superscript II version of RNase H⁻ Moloney murine leukemia virus [MoMLV] reverse transcriptase [Life Technologies, Inc., Gaithersburg, Md.] per μl). The suspension was divided into five aliquots of 75 μl each in thin-walled tubes (MicroAmp Reaction Tube; Perkin-Elmer) and was immediately transferred to a thermal cycler (Gene Amp PCR System 9600; Perkin-Elmer) that was preheated to 50°C for a hot start (10). The thermocycling format used for RT-PCR was as follows: one cycle of RT at 50°C for 10 min, followed by denaturation at 94°C for 2 min; 40 amplification cycles with denaturation at 94°C for 15 s, annealing at 50°C for 1 min, and extension at 72°C for 3 min; and a final incubation at 72°C for 7 min, followed by cooling at 4°C. The amplification products were separated from the latex particles by centrifugation at about 16,000 × *g* for 3 min, and 10 μl was analyzed by electrophoresis on a 0.8% (wt/vol) agarose gel, followed by staining with 0.5 μg of ethidium bromide (EtBr) per ml for 1 h. Water was used as the negative control that was treated by the same procedures described above.

In typical experiments for optimization of the reaction conditions, the RNA-oligo$(dT)_{30}$ hybrid derived from 10 μl of the starting RNA solution was suspended in 1 ml of RT-PCR buffer, dispensed into 20 to 40 tubes on ice, and then centrifuged and suspended in 75 μl of the RT-PCR mixtures which differed in their components. In the course of the optimization, when the concentrations of *Taq* and *Pwo* DNA polymerases had not yet been optimized, 0.075 U of the enzyme mixture (*Taq-Pwo*; at an unpublished ratio) from the Expand Long Template PCR system (Boehringer Mannheim Corp.) per μl was used in the experiments described below (Fig. 1A and Fig. 2A through D).

Southern hybridization. The 3-kb products of the RT-PCR were confirmed by Southern hybridization by using the six digoxigenin-labeled oligonucleotides comprising the four probe sets (P1-A, P1-B, P2-A, and P2-B) as described previously (3). For the P1-A strains, three probes (probes SR63d, SR65d, and SR69d) comprising the P1-A probe set were used together for strains S2 to S5, while the single probe SR65d was used for strain S1.

Analysis of single-stranded cDNA. The RT-PCR products showing aberrant migration patterns were analyzed for susceptibility to digestion by using mung bean (MB) nuclease (Life Technologies, Inc.) and for the possibility of making a transition to the 3-kb duplex form by reassociation treatments after denaturation. The amount of the RT-PCR product, pooled from 20 tubes and ethanol precipitated, was adjusted to approximately 3 μg per 128 μl of a test solution (34 mM KCl and 2 mM $MgCl_2$) and was subjected to two continuous cycles of denaturation-quenching treatments: the first cycle, heating at 95°C for 10 min, followed by immediate quenching in ice-water (rapid cooling); and the second cycle, heating at 95°C for 10 min, followed by gradual quenching from 80 through 40°C over a period of 13 h (slow cooling). Before and after each step of denaturation, five aliquots of 8 μl of product were collected from the test solution. Reaction with 0, 0.01, 0.1, 1, or 10 units of MB nuclease was conducted in 10 μl of solution containing 100 mM sodium acetate (pH 5.0), 1 mM zinc acetate, 500 mM NaCl, 10 mM L-cysteine, and 50% (vol/vol) glycerol at 37°C for 30 min. The reaction products were analyzed by agarose gel electrophoresis and Southern hybridization as described above.

Detection threshold. The RNA-oligo$(dT)_{30}$ hybrid, derived from 10 μl of the starting RNA and suspended in 1 ml of the RT-PCR buffer, was subjected to a 10-fold serial dilution (500 μl/dilution) by using the RT-PCR buffer as the diluent. After centrifugation, the precipitate from each dilution was suspended in 185 μl of the reaction mixture, dispensed into duplicate thin-walled tubes (75 μl/tube), and subjected to the RT-PCR, followed by agarose gel electrophoresis and Southern hybridization as described above. The reaction mixture without the RNA-oligo$(dT)_{30}$ hybrid was used as the negative control for RT-PCR and Southern hybridization.

RESULTS

Difficulties in initial attempts. Our initial attempts to develop a method for the amplification of a 3-kb region from the RNA polymerase gene to the 3′ poly(A) tail were based on reaction conditions similar to those used to amplify a small region in the RNA polymerase gene (3, 4). In these procedures, avian myeloblastosis virus (AMV) reverse transcriptase was used for the first-strand cDNA synthesis and the temperature for the extension step in PCR was 60°C. We sometimes could obtain the 3-kb product by using these reaction conditions. However, the amount of the product was very small (Fig. 1A, lanes 1 and 8), and the results were often hardly reproducible. The difficulties in amplification of the 3-kb region were not ascribed to an inability of AMV reverse transcriptase to transcribe a long region, nor were they due to a significant reduction in activity of *Taq* and *Pwo* DNA polymerases when they were used at 60°C instead of the commonly recommended 72°C (34), because RT-PCR yielded a large amount of internal products, including a 2.4-kb product spanning a region from the poly(A) tail to the 5′ end of ORF2 (Fig. 1A, lanes 2, 3, 9, and 10). We found that our difficulties were consistently linked to the production of cDNA fragments with an unexpectedly small size (Fig. 1A, left, lanes 1 and 4 to 8). These small products were not a result of primer oligomerization or contamination of nontarget nucleic acid because they specifically hybridized with the probe derived from the RNA polymerase region (Fig. 1A, right, lanes 1 and 4 to 8). The truncated RT-PCR products could result from either a mispriming of the VN primer due to its hybridization with an adenosine-rich site(s) on the template or a deletion of sequence due to incorrect polymerization across the bases of an intrastrand hairpin-like secondary structure (7, 38).

Effect of primer composition. The difficulties that we experienced in our initial attempts were overcome by the procedures described in Materials and Methods (Fig. 1B, lane 1). We found the use of the RNA-oligo$(dT)_{30}$ hybrid on the latex particles in the RT step to be extremely important, as demonstrated by complete elimination of the 3- and 2.4-kb products when the RNA-oligo$(dT)_{30}$ hybrid was denatured immediately before RT (Fig. 1B, lanes 6 to 9). Similarly, the importance of using the VN primer in the PCR step was clearly demonstrated by the absence of the 3-kb product when the VN primer was substituted by the TT primer (Fig. 1B, lane 2). However, substitution of the VN primer by the TT primer did not result in a substantial reduction in the yield of the 2.4-kb product (Fig. 1B, lanes 3 and 4). The differences between the results obtained with the VN primer and the TT primer suggest a strong secondary structure in a region between the 2.4- and 3-kb nucleotides from the poly(A) tail, a region between the RNA polymerase gene and the 5′ end of ORF2.

In addition, the 3-kb product was consistently accompanied by a faintly staining product, corresponding to 2.5 kb, that migrated relatively faster (Fig. 1B, left, lane 1). Unexpectedly, the hybridization signal of the faint product was comparable in intensity to that of the 3-kb product (Fig. 1B, right, lane 1). We also found that the substitution of the VN primer by the TT primer resulted in production of a cDNA fragment with the same migration pattern that could be detected only by Southern hybridization (Fig. 1B, right, lane 2). The product at the 2.5-kb position was digested by the MB nuclease (0.1, 1, and 10 U/μl) and was converted to a MB nuclease-resistant 3-kb duplex form by slow cooling after denaturation (data not shown). The results from this analysis suggest that the product at the 2.5-kb position represents the two complementary 3-kb single-stranded DNAs (ssDNAs), the accumulation of which is a result of self-annealing of each strand of the 3-kb cDNA by rapid cooling between denaturation and primer annealing in the PCR step. Of note, optimum RT-PCR conditions for the 3-kb product were quite different from those for the short products of the RNA polymerase region (3), since our method did not yield a 123-bp product visible by EtBr staining (Fig. 1B, lane 10). This fact explains why our new RT-PCR method resulted in the accumulation of the 3-kb ssDNA fragments rather than the truncated double-stranded DNA (dsDNA) that was a source of difficulties in our initial attempts. Besides the product at the 2.5-kb position, we sometimes noticed by Southern hybridization another product detectable at about the 5-kb position (e.g., Fig. 1B, right, lane 1). This product was also susceptible to MB nuclease digestion and shifted from the 5- to 3-kb position by slow cooling after denaturation (data not shown), suggesting that this product represents two dimers, each of which comprises two 3-kb fragments of ssDNA, with polarities that are the same within a dimer but complementary

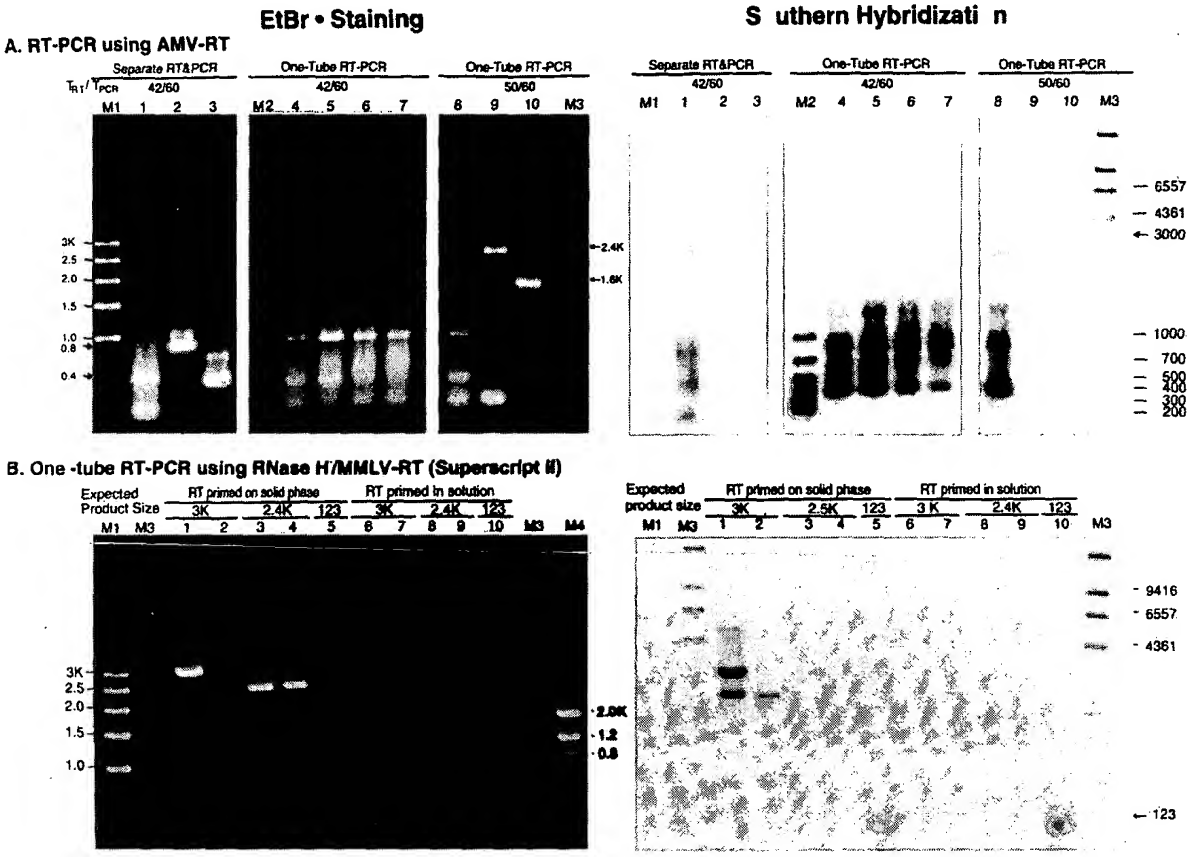

FIG. 1. Effect of primer composition. Poly(A)$^+$ RNA, purified with oligo(dT)$_{30}$-Latex, was used as the template. (A) Amplification products from RT-PCR in which AMV reverse transcriptase (Molecular Genetic Resources, Tampa, Fla) was used in the RT step. Lanes 1, 2, and 3, RT and PCR performed separately under the reaction conditions similar to those described previously (4), with the following modifications: for the primers used in the RT and PCR steps, 300 nM VN primer in combination with 300 nM (each) SR48, SR50, and SR52 (lane 1), SR62 (lane 2), or SR60 (lane 3); for the RT step, at 42°C for 1 h in 20 μl; and for the PCR step, 2 mM $MgCl_2$ and 0.2 mM (each) dNTP and 40 amplification cycles with denaturation at 94°C for 15 s, annealing at 42°C for 1 min 30 sec, and extension at 60°C for 5 min. Before and after the RT step, RNA was denatured at 90°C for 1 min in the presence of 5% (vol/vol) dimethyl sulfoxide and digested with 0.2 U of RNase H per μl at 37°C for 30 min, respectively. Lanes 4 to 7, one-tube RT-PCR performed under the reaction conditions similar to those reported recently (3), with the following modifications: for the primers, 310 nM (each) VN primer and SR48; for the concentration of $MgCl_2$, 1.75 mM (lane 4), 2.00 mM (lane 5), 2.25 mM (lane 6), or 2.50 mM (lane 7); for the DNA polymerase, 0.035 U of the *Taq-Pwo* enzyme mixture (Boehringer Mannheim) per μl; for the thermocyle format, one cycle of RT at 42°C for 45 min, followed by denaturation at 94°C for 2 min; 40 amplification cycles with denaturation at 94°C for 15 s, annealing at 42°C for 1 min 30 s, and extension at 60°C for 10 min; and a final incubation at 68°C for 7 min. Lanes 8 to 10, the conditions were similar to those for the one-tube RT-PCR, with the following modifications: for the primer combination, 310 nM (each) VN primer and SR48 (lane 8), VN primer and SR34 (lane 9), or Mon189 and SR34 (lane 10); for the RT step, at 50°C for 15 min and annealing in the PCR step at 50°C for 1 min and 30 s. The expected product sizes for lanes 1 and 4 to 8 were 3 kb, and those for lanes 2, 3, 9, and 10 were 0.8, 0.4, 2.4, and 1.6 kb, respectively. T_{RT}/T_{PCR}, temperatures of primer extension in the RT and PCR steps, respectively; 3K, 3-kb DNA. (B) One-tube RT-PCR with the Superscript II version of MoMLV reverse transcriptase in the RT step. Lanes 1 to 5, the components of the reaction mixture and the thermocycle conditions for RT-PCR are described in Materials and Methods, except for the primers used in lanes 2 to 5; lanes 6 to 10, products from a similar method with a modification in which the RNA-oligo(dT)$_{30}$ hybrid, formed on the surfaces of the latex particles, was denatured at 94°C for 3 min immediately before RT-PCR and the enzymes were added to the reaction mixture after denaturation. The primer pairs used were as follows: VN primer and SR48 (lanes 1 and 6), TT primer and SR48 (lanes 2 and 7), VN primer and SR34 (lanes 3 and 8), TT primer and SR34 (lanes 4 and 9), and SR33 and SR48 (lanes 5 and 10). (A and B) Lanes M1 to M4, DNA molecular markers, as follows: lane M1, DNA ladder XII (Boehringer Mannheim); lane M2, digoxigenin-labeled marker XI (Boehringer Mannheim); lane M3, digoxigenin-labeled marker II (Boehringer Mannheim); and lane M4, low-molecular-mass DNA ladder (Life Technologies). The amount of 3-kb dsDNA in lane M1 corresponds to 100 ng.

between the dimers. These two dimers might hybridize with each other at the region of the strong secondary structure, as predicted above.

Effects of other parameters. In addition to the primer components, we systematically examined the following parameters to optimize the reactions: pH of the RT-PCR mixture, concentrations of chemical and enzyme components (KCl, $MgCl_2$, dNTPs, primers, cosolvents [Triton X-100, Tween 20, Nonidet P-40, dimethyl sulfoxide, glycerol], MoMLV-reverse transcriptase, and DNA polymerases [*Taq*, *Pwo*]), and the thermocycling formats. Representative results from these experiments indicated that the optimum combination of $MgCl_2$ and dNTP concentrations depended on the primer extension temperature in the PCR step (Fig. 2A, left), a visible product was not obtained in the absence of nonionic detergents (Fig. 2B, left), and the optimum pH (based on 50 mM Tris-HCl at room temperature) and temperature in the RT step were 8.7 and 50°C, respectively (Fig. 2C, left). Similarly, MoMLV reverse transcriptase at a range of from 3.0 to 4.0 U/μl and the mixture of *Taq* and *Pwo* polymerases (unpublished ratio; from Expand

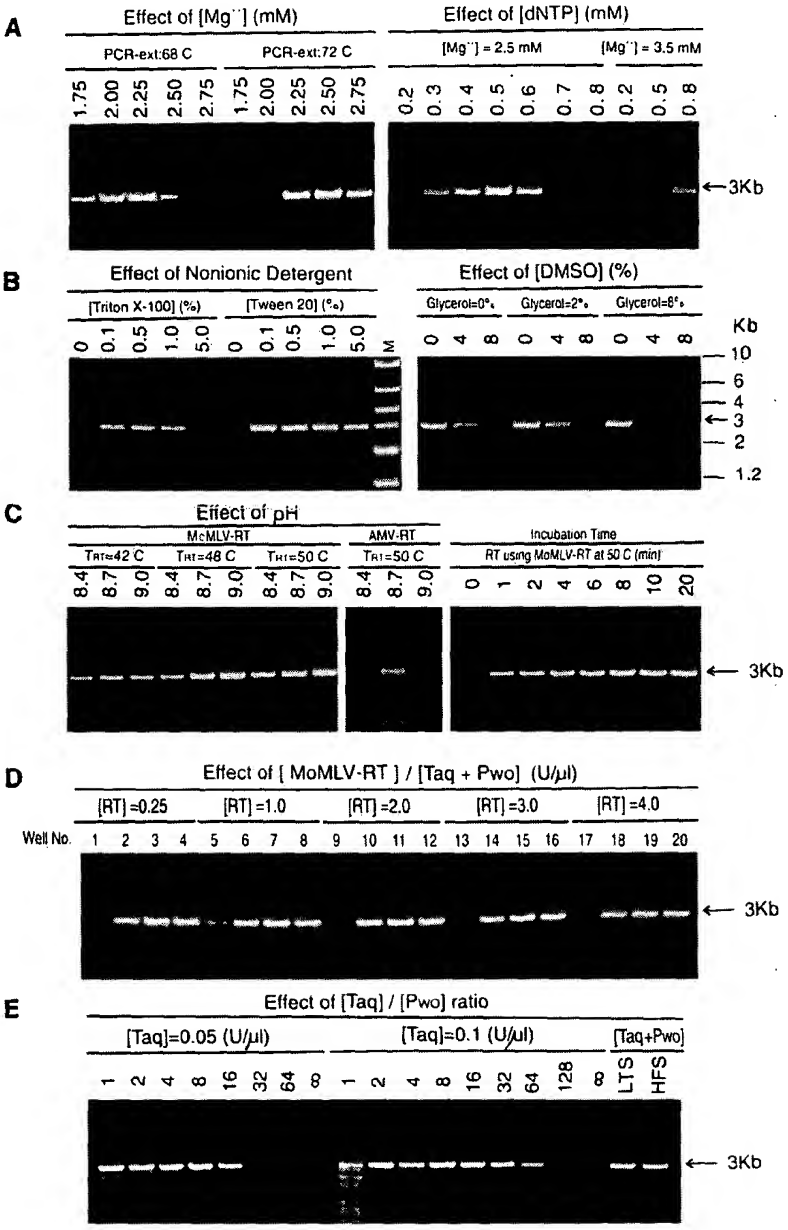

FIG. 2. Results of optimization of RT-PCR conditions. The reaction was run under the conditions described in Materials and Methods except for the parameters indicated. (A) Effect of concentrations of $MgCl_2$ and dNTPs. In the left gel, the $MgCl_2$ concentration was changed under the conditions in which each dNTP was present at 0.5 mM. Extension in the PCR step was performed at 68°C for 5 min or 72°C for 3 min. In the right gel, the dNTP concentration was changed under the conditions in which the $MgCl_2$ concentration was 2.5 or 3.5 mM and extension in the PCR step was performed at 72°C for 3 min. (B) Effect of nonionic detergents, dimethyl sulfoxide (DMSO), and glycerol. The effect of nonionic detergent was examined in the absence of dimethyl sulfoxide and glycerol (left gel), while the effects of dimethyl sulfoxide and glycerol were examined in the presence of 0.5% (vol/vol) Triton X-100 (right gel). M, a mixture of a high-molecular-mass DNA ladder and a low-molecular-mass DNA ladder (Life Technologies). The amount of 3-kb dsDNA marker corresponds to 60 ng. (C) Effect of pH of the reaction mixture and incubation temperature and time in the RT step. RT was carried out by using 3 U of the Superscript II version of MoMLV reverse transcriptase (left and right gels) per μl or 0.2 U of AMV reverse transcriptase (Molecular Genetic Resources, Tampa, Fla.) per μl (middle gel). (D) Effect of MoMLV reverse transcriptase/*Taq* plus *Pwo* ratio. [Taq+Pwo], concentration of an enzyme mixture (*Taq* and *Pwo* DNA polymerase, at an unpublished ratio) included in a commercially available PCR kit (Expand Long Template PCR System; Boehringer Mannheim): 0.025 U/μl, wells 1, 5, 9, 13, and 17; 0.05 U/μl, wells 2, 6, 10, 14, and 18; 0.075 U/μl, wells 3, 7, 11, 15, and 19; and 0.1 U/μl, wells 4, 8, 12, 16, and 20. (E) Effect of *Taq*/*Pwo* ratio. The *Taq*/*Pwo* ratio was changed from 1 to 64 or 128 by changing the concentration of the *Pwo* DNA polymerase under conditions in which the concentration of *Taq* DNA polymerase was 0.05 U/μl (left eight lanes) or 0.1 U/μl (middle nine lanes). ∞, absence of the *Pwo* DNA polymerase. LTS and HFS, 0.075-U/μl enzyme mixtures included in the Expand Long Template PCR System and Expand High Fidelity PCR System (Boehringer Mannheim), respectively.

Long Template PCR system) at a range of from 0.05 to 0.075 U/μl gave the best results among the combinations examined (Fig. 2D). Although MoMLV reverse transcriptase showed an inhibitory effect on *Taq* and *Pwo* DNA polymerases (e.g., Fig. 2D, lanes 6 and 7 versus lanes 18 and 19), it was much less prominent than that reported for AMV reverse transcriptase (36). Under the conditions in which MoMLV reverse transcriptase was 3 U/μl, neither *Taq* polymerase nor *Pwo* polymerase alone could produce a visible amount of the 3-kb cDNA fragment (Fig. 2E, lane ∞, for *Taq* polymerase; data not shown for *Pwo* polymerase). The optimum *Taq*/*Pwo* ratio determined in this study was 8 to 16, which may be comparable to those used in the enzyme mixtures (*Taq* and *Pwo*; at an unpublished ratio) included in the commercially available kits (Fig. 2E, right).

One striking finding of the optimized reaction conditions was the extremely fast production of the first-strand cDNA during the RT step: the 3-kb product became clearly visible by EtBr staining after a 1-min incubation at 50°C, reached steady state by 8 to 10 min, and did not increase with an incubation time of ≥20 min (Fig. 2C, right; data not shown for 30 min). The rapid production of the 3-kb cDNA fragments was solely ascribed to the activity of MoMLV reverse transcriptase (data not shown), not to the cooperative function of MoMLV reverse transcriptase and *Taq* polymerase, which might exhibit RT activity (37, 41).

Of note, the better results were obtained from the use of freshly prepared RNA (data not shown). In addition, the following procedures were of particular importance among the procedures described in Materials and Methods (data not shown): (i) prepare the RT-PCR mixture by adding *Taq* and *Pwo* DNA polymerases and MoMLV reverse transcriptase immediately before it was mixed with the RNA-oligo$(dT)_{30}$ hybrid, (ii) keep the reaction mixture at 0°C before and after mixing with the hybrid, and (iii) hot start the RT within 30 min after the addition of MoMLV reverse transcriptase to the reaction mixture.

Detection threshold. The detection threshold of this method was estimated to be 0.2 ng of the starting RNA, which resulted in a 3-kb product barely visible by EtBr staining but clearly detectable by Southern hybridization (Fig. 3). The 3-kb ssDNA, migrating faster than the double-stranded product, was consistently detected by Southern hybridization (Fig. 3B). The background ladder detected by Southern hybridization with 20 ng of the starting RNA may be an artifact associated with an excess amount of latex particles (250 μg/reaction) to which the template-primer-enzyme complex may stick (16).

Application of the new method. Amplification of the remaining 23 SRSV strains by using the optimized conditions resulted in 3-kb products clearly visible by EtBr staining (Fig. 4). This collection included 4 P1-A, 4 P1-B, 5 P2-A, and 10 P2-B strains. The specificity of the products was confirmed by Southern hybridization, which also detected the ssDNA product migrating at about 2.5 kb (data not shown). The negative control consisting of water did not show the specific product (data not shown).

A success rate of amplification of the 3-kb genome region of SRSVs was about 75% upon further application of this method to RNAs prepared from stool specimens from individuals involved in 43 outbreaks, including 24 outbreak specimens used in this study (data not shown). The unsuccessful amplification resulted from the RNAs which were kept at −70°C for 6 months to 3 years, suggesting degradation of RNA during the storage.

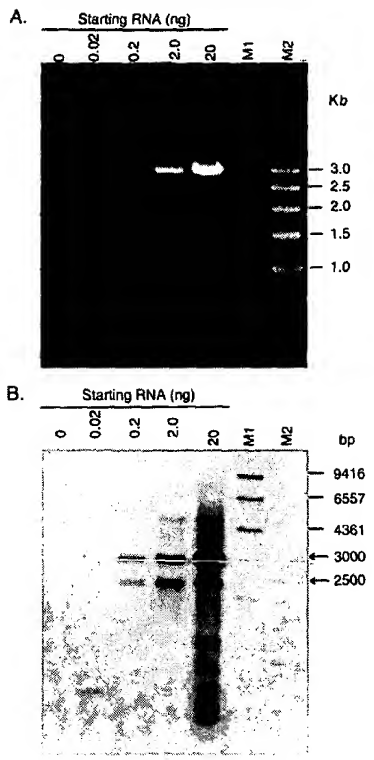

FIG. 3. Detection threshold of the one-tube RT-PCR method. The procedures are described in Materials and Methods. Lanes M1 and M2, digoxigenin-labeled DNA molecular marker II and DNA marker XII (Boehringer Mannheim), respectively. The amount of the 3-kb marker in lane M2 corresponds to 100 ng.

DISCUSSION

In the past 3 years, portions of the RNA polymerase gene from many strains of SRSV have been sequenced on the basis of the RT-PCR products derived from this region (3, 4, 11, 15, 19, 33, 40, 44). However, efforts to use these sequences for positive-sense primers to amplify a 3-kb region from the RNA polymerase gene to the poly(A) tail have largely been unsuccessful, delaying progress in the genetic and antigenic characterization of SRSVs. The difficulties might have been associated with the secondary structure within the target sequence, the use of oligo(dT) primers with low priming efficiency (6, 28), the relatively large size of the amplification target requiring highly processive polymerization, and low concentration of the highly purified template RNA available from stool samples. The results of the present study consistently suggested that the secondary structure is the most problematic among the factors making amplification of the 3-kb RT-PCR product difficult. We have developed a method which overcomes these difficulties and which allows for the efficient and reproducible amplification of the 3-kb region from stool samples containing a diverse set of SRSV strains.

The precise mechanisms underlying the high efficiencies of RT and PCR have not been analyzed in this study. In effect, the efficiency of RT in this method could follow from an increased efficiency of RT initiation, a high rate of strand extension, and, probably, a high processivity (number of bases incorporated during a single round of primer extension) of RNase H^- MoMLV reverse transcriptase. Our particularly important

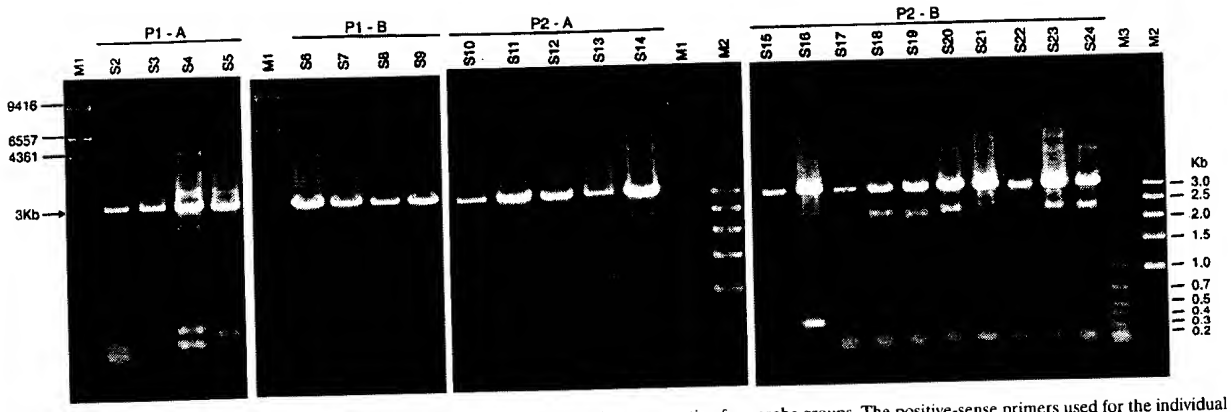

FIG. 4. Results of application of the one-tube RT-PCR method to SRSV strains representing four probe groups. The positive-sense primers used for the individual strains are indicated in Table 1. Lanes M1 to M3, DNA molecular markers, as follows: lane M1, digoxigenin-labeled marker II (Boehringer Mannheim); lane M2, DNA ladder XII (Boehringer Mannheim); and lane M3, digoxigenin-labeled marker X1 (Boehringer Mannheim). The amount of the 3-kb dsDNA marker in M2 corresponds to 100 ng.

finding demonstrated the need for conducting RT on the solid surfaces of latex particles rather than in solution, and this step most likely resulted in the formation of a stable enzyme-template-primer complex on the surfaces of the latex particles. The mechanism of this phenomenon may be comparable to that reported for the initiation of avian retrovirus reverse transcriptase, for which an enzyme-template-tRNA primer complex is required to be maintained in a specific orientation, albeit in a solution and not on the solid phase (1). Our additional finding that a relatively large amount of the first-strand cDNA needed for the 3-kb amplicon was produced with 1 min of incubation at 50°C indicates that the chain elongation rate of RT by this method is at least 50 nucleotides per second, a rate about 10-fold higher than that reported for MoMLV reverse transcriptase (43). This extremely high transcription rate may be associated with the increased temperature of 50°C that would result in the stimulation of transcription activity of the enzyme and partial disruption of the secondary structure. It is also possible that this high transcription rate is an intrinsic characteristic of the Superscript II version of RNase H^- MoMLV reverse transcriptase (43). The temperature and the incubation time established for the RT step in this study are comparable to those used in a one-tube RT-PCR with AMV reverse transcriptase reported for human immunodeficiency virus type 1 mRNA (30). However, the reaction conditions of pH 8.7, a temperature of 50°C, and an incubation time of 10 min optimized for MoMLV reverse transcriptase in our method are quite different from those recommended by the company supplying this enzyme (32). In our study, MoMLV reverse transcriptase molecules were not sequestered away from the transcript immediately upon their first dissociation. Therefore, we could not determine the processivity of this enzyme. Nevertheless, the finding of the extremely fast production of a relatively long product described above suggests that the processivity of this enzyme must be very high under these conditions for RT. Taken together, the amount of product in the RT step of this method might reach a level that could not have been achieved by the RT procedures of the RT-PCR methods routinely used to amplify the RNA polymerase region of SRSVs.

In the PCR step of this method, the high efficiency of amplification for the 3-kb region may be mainly associated with the negative-sense primers and the two DNA polymerases. Our results indicated the importance of using a mixture of 12 combinations of phased oligo$(dT)_{25}$ primers (VN primer) as compared with the commonly used oligo$(dT)_{27}$ primer (TT primer). These results confirm the importance of the two 3′ bases, VN, in VN primers for the efficient initiation of primer extension from the 3′ poly(A) tail, as reported by other researchers (6, 28). Similarly, our results indicated that the combined use of *Taq* and *Pwo* DNA polymerases is crucial for amplification of the 3-kb region. These results suggest that a reciprocal action of *Taq* polymerase with high processivity and *Pwo* polymerase with proofreading activity (3′ to 5′ exonuclease activity) is required to effectively transcribe this target region with a stable secondary structure, as has been reported for amplification of long targets from bacteriophage and human genomic DNAs (5, 9). Of note, our finding that the optimum combination of $MgCl_2$ and dNTP concentrations depends on the primer extension temperature is an additional element that allowed us to optimize the thermocycle conditions for amplification of the 3-kb product.

Using this new method, we could amplify the 3-kb region from the 24 SRSV strains representing four probe groups (3). The amount of the RT-PCR products was high for all 24 strains examined and sufficient for future studies of cloning, sequencing, and expression of the capsid protein and the protein of unknown function encoded by ORF3. While SRSVs have not yet been cultivated in vitro, this novel method has a great advantage over other RT-PCR methods in that its use precludes the possibility of mistaking the sequences derived from different strains for a single origin. Such mistakes may happen in a dual or mixed infection of SRSVs (2, 40), when two different genome regions (e.g., the RNA polymerase and the viral capsid regions) are amplified by separate RT-PCR assays. In addition, the amount and purity of the starting RNA required in this method are comparable to, or less than, those required in the RT-PCR methods currently used for the diagnosis of SRSV infections (3, 12, 14, 20). Moreover, the use of the universal VN primer precludes the necessity to newly design the negative-sense primers from the sequences in ORF3 or the 3′ untranslated region that may be more diverse than those in the RNA polymerase region (8, 13, 19, 24). In summary, the availability of this novel method should facilitate the molecular characterization of SRSVs, especially in genetic and antigenic relationships, to provide a firm scientific foundation for improvements and refinements of SRSV diagnostics. It is possible that this method may be useful in the study of not only other single-stranded RNA viruses but also eukaryotic mRNAs

(28), in which amplification of a long genome region has been difficult because of the secondary structure or the small amount of the template RNA available.

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